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THE UNIVERSITY OF ALBERTA
STUDIES ON BOVINE PROTHROMBIN

by



RONALD KEITH BULL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies and
Research, for acceptance, a thesis entitled "STUDIES ON
BOVINE PROTHROMBIN" submitted by RONALD KEITH BULL in
partial fulfilment of the requirements for the degree of
Master of Science.

ABSTRACT

Prothrombin is a glycoprotein important in the coagulation of blood. It is the inactive precursor of thrombin (EC 3.4.4.13), the enzyme responsible for the hydrolysis of fibrinogen to fibrin. Conversion of prothrombin to thrombin requires the simultaneous presence of calcium ions, phospholipids, and two other coagulation factors, factor V and activated factor X.

Work reported in this thesis includes the development of a method of prothrombin preparation, determination of some of the physico-chemical properties of the protein, and investigation of the nature of Ca^{+2} -dependent prothrombin-phospholipid complex formation.

Prothrombin was prepared from bovine plasma by adsorption onto BaSO_4 followed by elution with sodium citrate. The eluate was further purified by ammonium sulphate fractionation, DEAE-cellulose ion exchange chromatography and Sephadex G-150 gel filtration. The inclusion of diisopropyl phosphorofluoridate in all buffer and protein solutions was found to prevent degradation of prothrombin during the preparation.

The final product was demonstrated to be homogeneous by disc electrophoresis on polyacrylamide gel, analytical ultracentrifugation and gel filtration. The prothrombin obtained was free of thrombin and detectable factor X. Analytical ultracentrifugation studies indicated $S_{20,w}^0$

and $D_{20,w}^0$ values of 5.03 S and 5.22 Fick units respectively. Molecular weight using the Svedberg equation was 79,000. Equilibrium \bar{M}_w determinations yielded values of 75,900 and 75,700 by the meniscus depletion method. Low-speed equilibrium ultracentrifugation data analysed as a function of concentration indicated evidence of protein aggregation at higher concentrations and yielded an extrapolated \bar{M}_w value of 67,500. The molecular weight of prothrombin by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis was 110,000 and 84,000 respectively. The final product was also characterized by amino acid analysis.

The ability of phospholipids to bind prothrombin in the presence of Ca^{+2} ions was studied as a function of lipid composition. Two methods were used to evaluate binding. The first method involved gel filtration on Sephadex G-200 at pH 9.0 to separate the lipid-bound and free prothrombin; the second involved precipitation of the complexes at pH 6.5. Phosphatidyl choline (PC) or a mixture of this lipid with phosphatidyl ethanolamine (PE) bound very little prothrombin, whereas mixtures of phosphatidyl serine (PS) with PC were very effective in binding and the complexes that formed were highly active in the one-stage prothrombin assay. PS alone, phosphatidic acid (PA) alone, PA:PS mixtures, and PA:PC mixtures containing high proportions of PA, bound prothrombin very effectively, but the complexes formed were much less active

than when PS:PC was used. Bound protein could be more readily removed from PS:PC than from PS:PA by treatment of the complexes with EDTA.

The presence of CaCl_2 produced changes in the $S_{20,w}$ concentration dependence and ultraviolet absorption spectrum of prothrombin indicating a Ca^{+2} -induced conformational change.

It is suggested that an optimal condition is required to reversibly bind prothrombin prior to conversion to thrombin. Anticoagulant effects ascribed to acidic phospholipids may be due to irreversible binding of clotting factors to lipid surfaces.

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LIST OF ABBREVIATIONS

A_{280}	absorbance at wavelength of 280 nm
A_{750}	absorbance at wavelength 750 nm
BSA	bovine serum albumin
$^{14}\text{C-PC}$	^{14}C labelled phosphatidyl choline
CPM	counts per minute
DFP	diisopropyl phosphorofluoridate
EDTA	disodium ethylenediaminetetraacetate
Factor Xa	activated factor X
Kd	gel filtration distribution coefficient
\bar{M}_w	weight-average molecular weight
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PS	phosphatidyl serine
SDS	sodium dodecyl sulphate
Ve	gel filtration elution volume
Vo	gel filtration void volume
Vt	gel filtration total column volume

CHAPTER I

INTRODUCTION

A. Prothrombin

Prothrombin is a plasma glycoprotein. It is the inactive precursor of thrombin, the enzyme responsible for the cleavage of fibrinogen to a fibrin monomer which subsequently polymerizes to form an insoluble clot. Prothrombin has been designated as Factor II by the International Committee on the Nomenclature of Blood Clotting Factors (1).

Barnhart, by using immunofluorescence techniques, has demonstrated that prothrombin is synthesized by the liver parenchymal cells of man (2) and a number of other mammals which have been examined (3, 4). The synthesis of prothrombin is vitamin K dependent (5). Although the specific action of the vitamin is uncertain, it does not appear to be a constituent of prothrombin (6) and appears to act at, or subsequent to, the translational level of synthesis (4, 7-9).

Structural knowledge of prothrombin is rather fragmentary. At present, most available information is related to bovine prothrombin. Human prothrombin does appear to have a similar amino acid composition, carbohydrate content and molecular weight (10). The total number of amino acids in prothrombin is estimated between 520 and 560 (11-13). These appear to be connected

in a single chain (13-15) with an N-terminal alanine and C-terminal serine (16, 17). Prothrombin is lacking in free sulphhydryl groups and reportedly contains eight moles of disulphide per mole (18). Molecular weight estimates range between 66,000 and 74,000 (12-15, 19). The carbohydrate portion of prothrombin represents 10% to 12% of the total molecular weight (10, 12, 20) and contains the oligosaccharide sequence sialic acid-galactose-mannose-glucosamine-peptide (20). The results of optical rotatory dispersion and circular dichroism studies indicate that prothrombin is predominantly a random-coil structure possibly containing a small amount of β -structure (10, 21, 22).

Thrombin (EC 3.4.4.13) is also a glycoprotein, containing 5% to 6% carbohydrate of composition similar to that of the zymogen (13). The molecular weight of thrombin is usually accepted as being approximately 34,000 (23, 24), although thrombin activity has been reported with preparations of molecular weights ranging from 8,000 (25) to 36,000 (26, 27). The amino acid compositions of prothrombin and thrombin are similar (11, 13).

Hartley and Magnusson (13, 28, 29), in preliminary reports on the primary structure of thrombin, have indicated that the enzyme is composed of two polypeptide chains, linked by a single disulphide bridge. The A-chain, which has been sequenced, consists of 49 amino acids, including threonine and arginine respectively as N- and C-termini. Sequencing of the B-chain appears to be almost

completed. The tentative amino acid sequence which has been published indicates that the B-chain is comprised of approximately 260 amino acid residues. It contains an N-terminal isoleucine and C-terminal serine. All of the carbohydrate is attached to an asparagine residue in the B-chain.

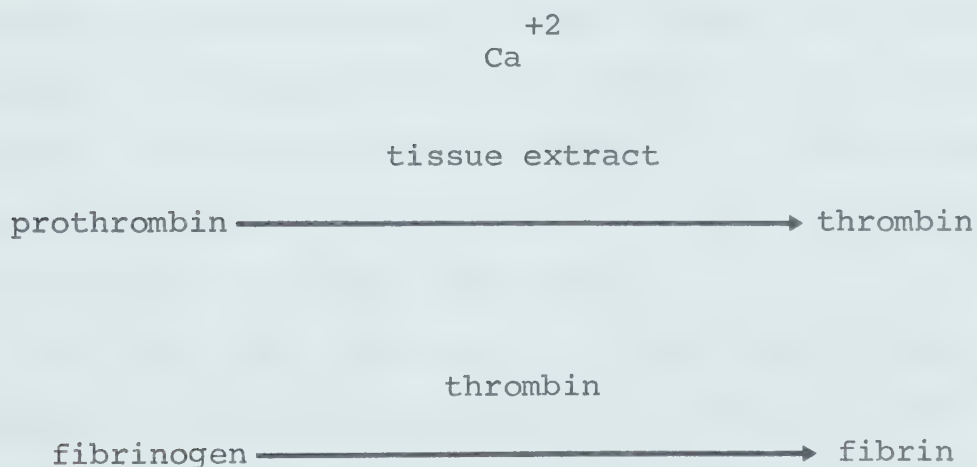
Thrombin is a serine proteinase (30, 31). The thrombin B-chain appears to have considerable sequence homology with trypsin, chymotrypsins A and B, elastin and papain. A number of the structural features of other serine proteinases occur in thrombin (13, 28). Homologous structures around histidine-57, aspartic acid-102 and active serine-195 (numbering corresponds to the chymotrypsinogen sequence) are compatible with the "charge relay system" proposed by Blow et al. (32) as well as the general acid-general base catalysis proposed by Polgar and Bender (33). The "internal activation salt bridge" suggested by Sigler et al. (34) also appears to be operative (13, 28). The "tosyl hole" structure (35) is also possible in thrombin: the residue at the bottom of the hole would be aspartic acid as in the case of trypsin. In addition, three of the four disulphide bridges common to chymotrypsinogen and trypsinogen are likely in thrombin. These include the "histidine loop", the "methionine loop" and the "serine loop". In thrombin the half-cystine residues of the "B-C chain" bridge are replaced by glycine and methionine.

Thrombin, like trypsin, hydrolyses arginyl and lysyl

bonds in synthetic esters (36, 37). In terms of natural substrates however, thrombin is more specific than trypsin. Fibrinogen monomer (MW 170,000) contains over 100 potential sites of tryptic cleavage, however thrombin acts at only six of these, all of which are arginyl-glycyl bonds (38). Thrombin also affects other coagulation factors. It activates factor XIII (39, 40) and enhances the activities of factors V (41-43) and VIII (44, 45). Thrombin also hydrolyses a platelet membrane protein (46, 47) and promotes platelet aggregation (48).

B. Coagulation Theories

The classical theory of coagulation, advanced by Morawitz in 1905 (49), described the formation of a fibrin clot in the two reaction sequences shown below.



The incidence of a number of naturally occurring coagulation disorders subsequently demonstrated the inadequacy of the four component theory. Attempts to isolate coagulant components to correct these deficiency diseases yielded both a proliferation of factors and a profusion of terminology. An International Committee, established to standardize terminology and eliminate duplications, adopted the convention of delineating each factor by a Roman numeral (1). Activated forms of these factors are generally designated by the subscript 'a'. This convention will be adhered to here, except in the cases of fibrinogen (factor I) and prothrombin (factor II). A list of coagulation factors, synonyms and corresponding deficiency diseases is presented in Table I (1, 50).

The formation of a fibrin clot in plasma in the presence of thromboplastin (tissue extract) and calcium was shown to be dependent on the presence of a fifth factor, factor V, reported by Owren in 1947 (51). The subsequently discovered factors VII (52) and X (53) were also demonstrated to be essential in this system (54).

The fact that blood clots in glass tubes without the necessity of tissue extract made it evident that an alternative coagulation pathway, the intrinsic system, existed (55). The tissue-extract pathway became referred to as the extrinsic system. The intrinsic prothrombin activation system was shown to be dependent on factors VIII, IX, XI and XII as well as the previously mentioned

TABLE I

COAGULATION FACTORS, SYNONYMS

AND CORRESPONDING DEFICIENCY DISEASES

Roman Numeral	Synonyms		Deficiency Diseases
	Common	Others	
I	Fibrinogen	-	Afibrinogenemia
II	Prothrombin	-	Hypoprothrombinaemia
III	Tissue thromboplastin	Extrinsic thromboplastin	-
IV	Calcium	-	
V	Ac-globulin, Proaccelerin	Labile factor, plasma accelerator globulin	Owren's disease, Parahaemophilia
VII	Proconvertin	Autoprothrombin I, stable factor	Congenital factor VII deficiency
VIII	Antihæmophilic globulin (AHG)	Antihæmophilic factor (AHF), platelet cofactor I, Antihæmophilic globulin A	Hæmophilia A
IX	Christmas factor	Autoprothrombin II, plasma thromboplastin component (PTC) platelet cofactor II Antihæmophilic factor B	Christmas disease, Hæmophilia A
X	Stuart-Prower factor	Autoprothrombin III	Stuart disease
XI	Plasma thromboplastin antecedent (PTA)	Antihæmophilic factor G	Congenital PTA deficiency
XII	Hageman factor	Contact factor, surface factor	Hageman trait
XIII	Fibrin stabilizing factor (FSF)	fibrinase Laki-Lorand factor	Factor XIII deficiency

factors V and X common to the extrinsic system (56, 57).

Attempts to systematize the interrelationships of these various factors resulted in the development of the "cascade" and "waterfall" theories of Macfarlane (58) and Davie and Ratnoff (59) in 1964. A revised version of the original cascade theory is shown in Fig. I.

Macfarlane has enumerated three propositions which form the basis of the cascade theory (61):

1. Normal prothrombin activation involves a number of functionally and perhaps physically distinct entities present in the plasma.
2. These entities react one upon the other in a certain preferential sequence during normal clotting.
3. Most, if not all, of these entities are proenzymes, each yielding an enzyme capable of activating the next component of the sequence.

Initiation of the clotting mechanism results in a series of zymogen-enzyme transitions which culminates in the formation of a fibrin clot. As pointed out by Macfarlane, one of the principal advantages of such a system is the photomultiplier or biochemical amplifier effect of such a sequence in responding to minute stimuli (58, 62). The first noted exception to the assumption that all coagulation factors are enzymes was factor V which appears to be a high molecular weight cofactor (63-66). Although not shown in Fig. 1, evidence suggests that factor VIII (67, 68) and possibly factor XI (69) may also be cofactors.

Figure 1. The cascade theory of blood coagulation (Reference 60).

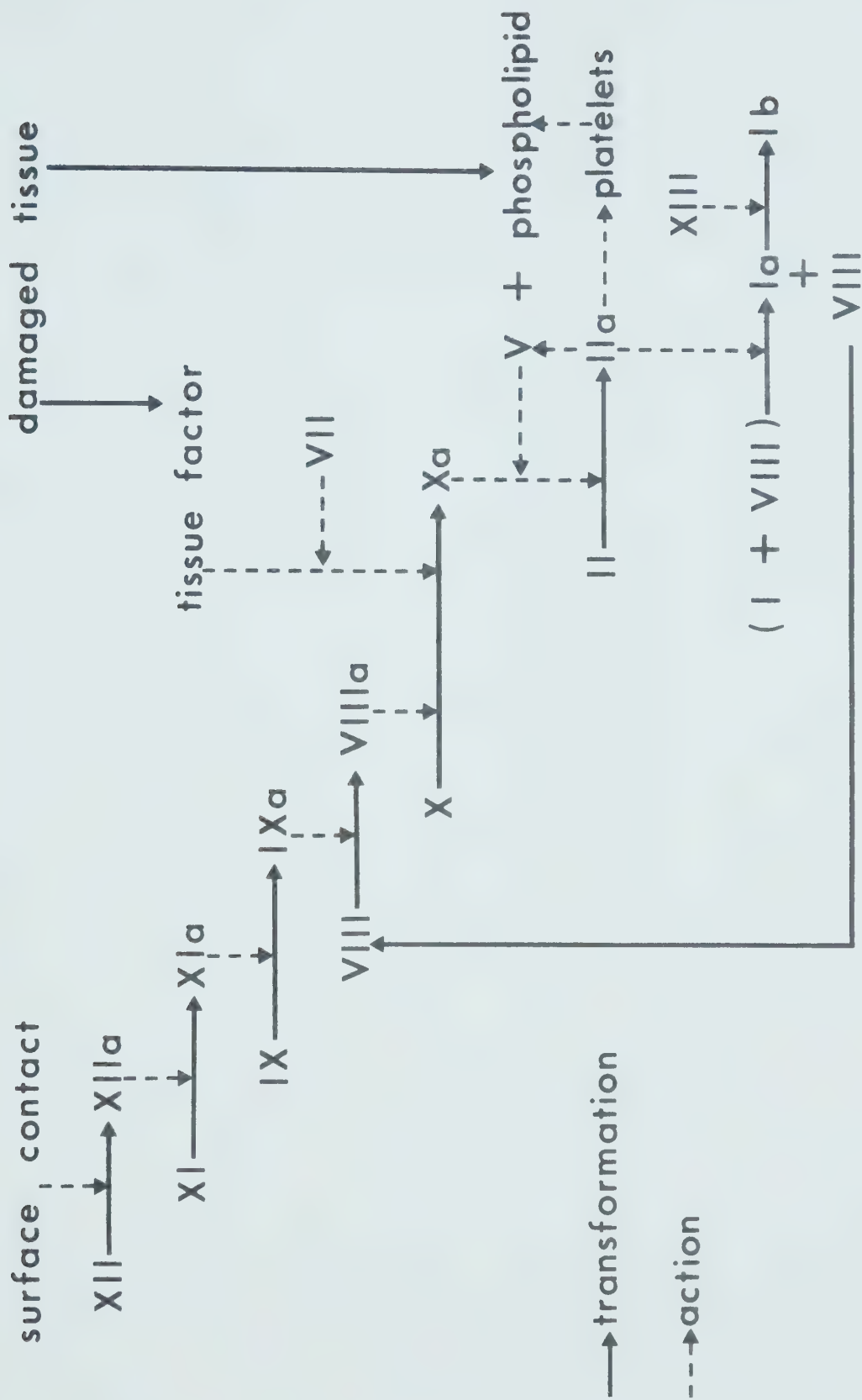
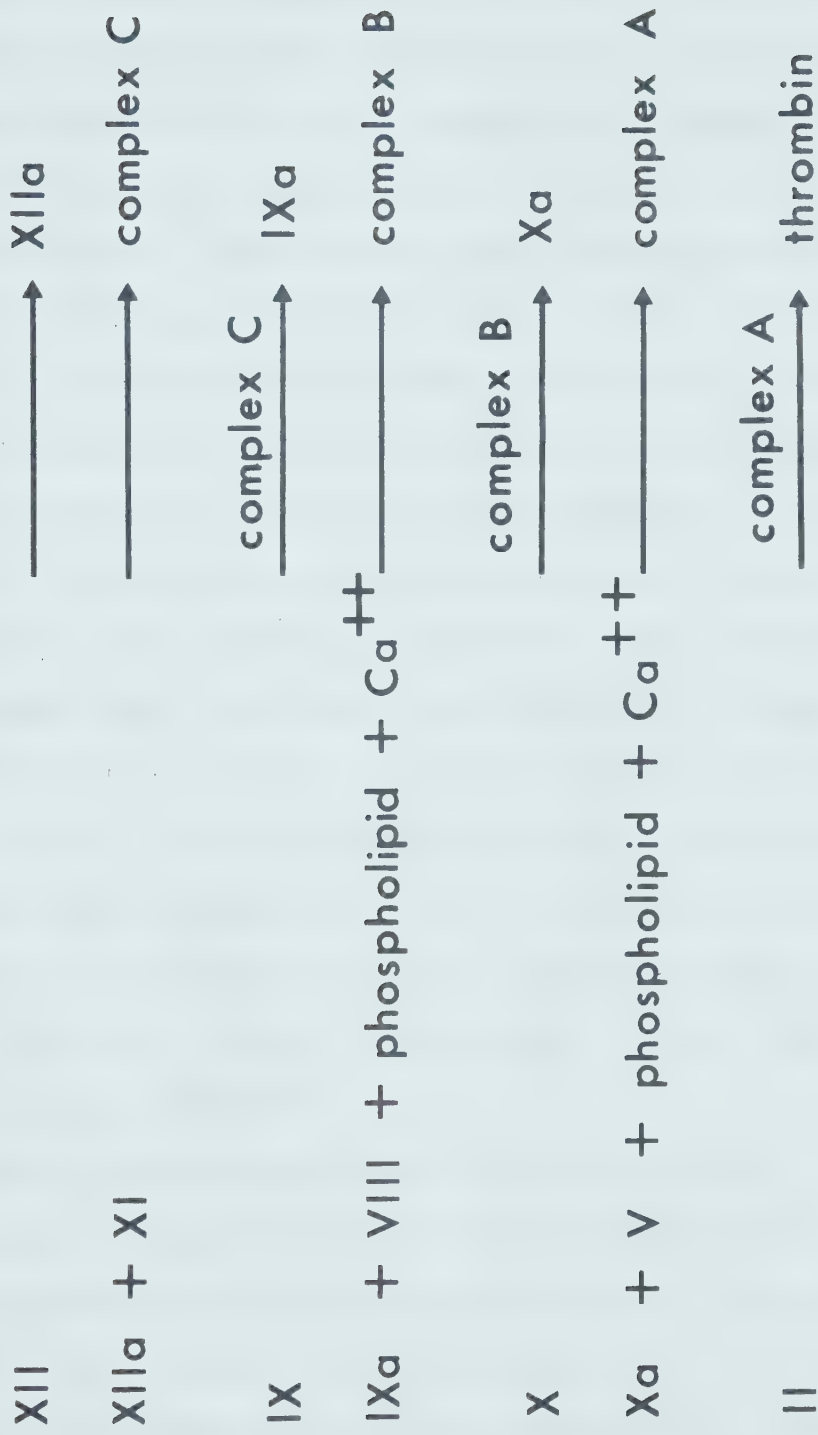


Figure 2. A sequential theory of blood coagulation which emphasizes complex formations (Reference 70).

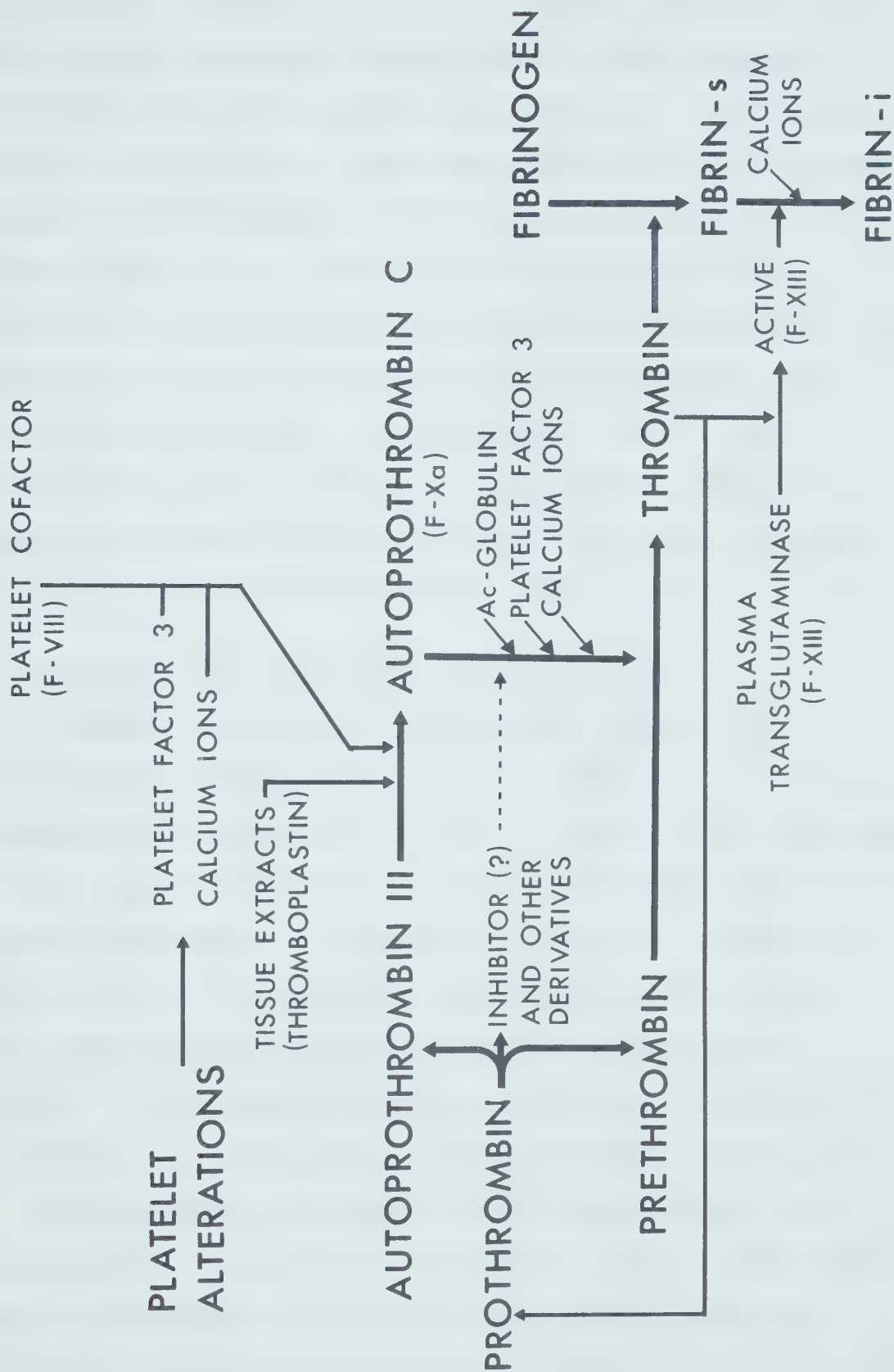


A reaction scheme proposed recently by Hemker et al. (70) is shown in Fig. 2. This scheme represents an extension of models suggested by Barton (71) and Hemker and Kahn (72). The sequence retains the essential features of an enzyme cascade but places emphasis on complex formation and the cofactor roles of factors V, VIII and XI.

Although all components in the sequence prior to factor X, with the exception of factor VIII, have been isolated in reasonably pure forms, evidence for their various interactions has been indirect and circumstantial. Current coagulation theories likely represent only a rough outline of the actual mechanisms involved. Sequential mechanisms, very similar in nature to those proposed for blood coagulation, have been postulated for a number of other biological systems. Wald has suggested such a scheme for the visual excitation process and has compared this with blood coagulation (73). Other examples include the formation of plasmin, kinin, complement and angiotensin (74). Factor XII appears to be common to the first two of these processes (74-76).

Another coagulation theory, the prothrombin derivatives theory, developed by W. H. Seegers (77, 78) differs somewhat from the cascade theories. The central feature of this theory is a multifunctional "prothrombin" molecule which contains inactive precursors of factors II, VII, IX and X described as prothrombin derivatives. An outline of the derivatives theory is shown in Fig. 3.

Figure 3. The prothrombin derivatives theory of W. H. Seegers (Reference 79).



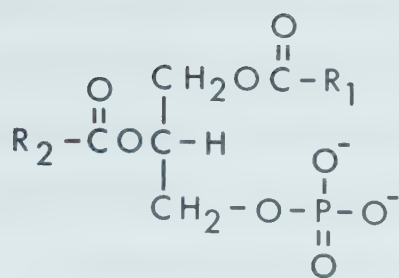
Although the terminology is more complex, the reactions which these components undergo are, in many respects, compatible with the cascade theory (80, 81). The disputed point of this theory is that the purity of the prothrombin prepared by the Seegers' method is questionable (82). In recent publications, proponents of this theory have indicated a gradual acceptance of the cascade approach in preference to the prothrombin derivatives concept (83). The term "prothrombin", which had been used by the derivatives group to describe a prothrombin preparation containing factors II, VII, IX and X, has been replaced by the term "prothrombin complex" (84).

C. Phospholipids and Prothrombin Activation

Optimal activation of prothrombin requires the simultaneous presence of factor V, factor Xa, Ca^{+2} and a phospholipid surface (66, 85, 86). Factor Xa is the enzyme in this system and it will, in relatively high concentration, convert prothrombin to thrombin on its own, although at a rate too low to be useful in haemostasis (87). Factor V is a cofactor and alone has no effect on prothrombin (63-66). The phospholipid surface is likely important in coordinating enzyme, cofactor and substrate (85, 88, 89).

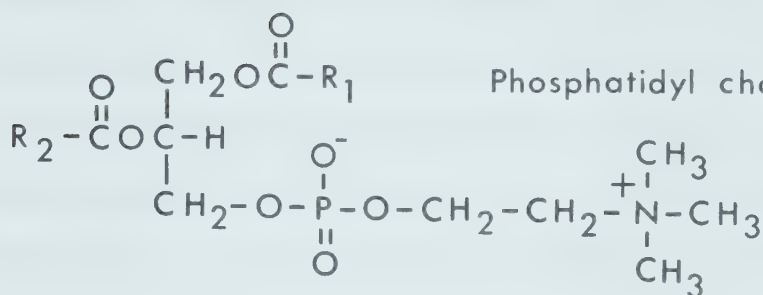
The chemical structures of four phospholipids are illustrated in Fig. 4. The hydrophilic polar substituents and the hydrophobic hydrocarbon side chains impart an amphipathic character to these compounds. In aqueous media,

Figure 4. Chemical structures of four phospholipid classes.

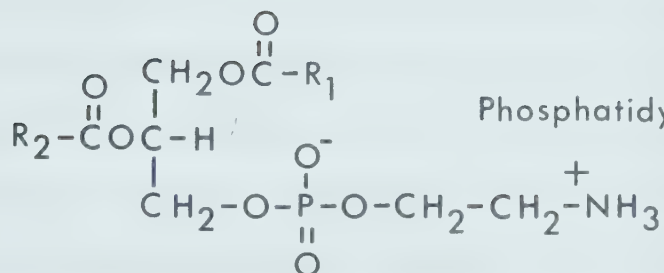


R_1, R_2 - hydrocarbon chains

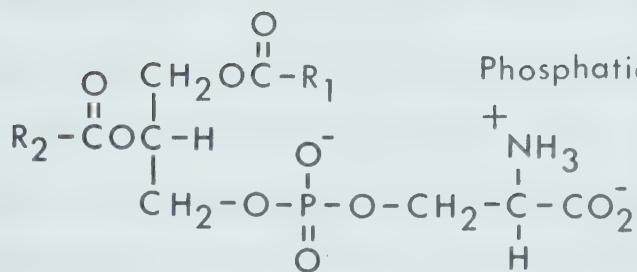
Phosphatidic acid (PA)



Phosphatidyl choline (PC)



Phosphatidyl ethanolamine (PE)



Phosphatidyl serine (PS)

phospholipid molecules form aggregates. The nature of these aggregates is influenced by phospholipid concentration, temperature, the length and degree of saturation of the hydrocarbon chains and the type of polar substituent.

Bangham (90) has outlined four reversible stages of aggregation which are shown in Fig. 5. Common to each stage is a spatial orientation of polar groups (represented by circles) in the aqueous phase which acts as an enclosure to the hydrophobic hydrocarbon chains (represented by lines). Although all four stages are theoretically possible, the existence of any particular stage depends on the actual lipid used. The first stage, which may exist at low lipid concentrations, is an alignment of molecules at an air-water interface. Saturation of the interface leads to bulk lipid aggregations. At the critical micellar concentration, isotropic spheres (micelles) are formed. The micelles of this second stage, which may be formed at concentrations of approximately 0.5% w/v, represent aggregates of up to 100 monomer units. The third stage may occur at concentrations of approximately 50% w/v and is a liquid crystalline phase. The lamellae formed in this stage are separated by the aqueous medium and are free to slide past one another. This phase can exist in equilibrium with the two preceding phases. A crystalline solid, the fourth stage, results when the partial volume of lipid exceeds 95%. It is similar to a lamellar phase in which the lamellae are fixed relative to each other.

Figure 5. Structural arrangements of amphipathic lipids at various concentrations in water (Reference 90). Polar groups are represented by circles. Lines represent hydrocarbon chains.

A - monolayer at air-water interface.

B - molecules in solution.

C - micelle formation at approximately
0.5% w/v.

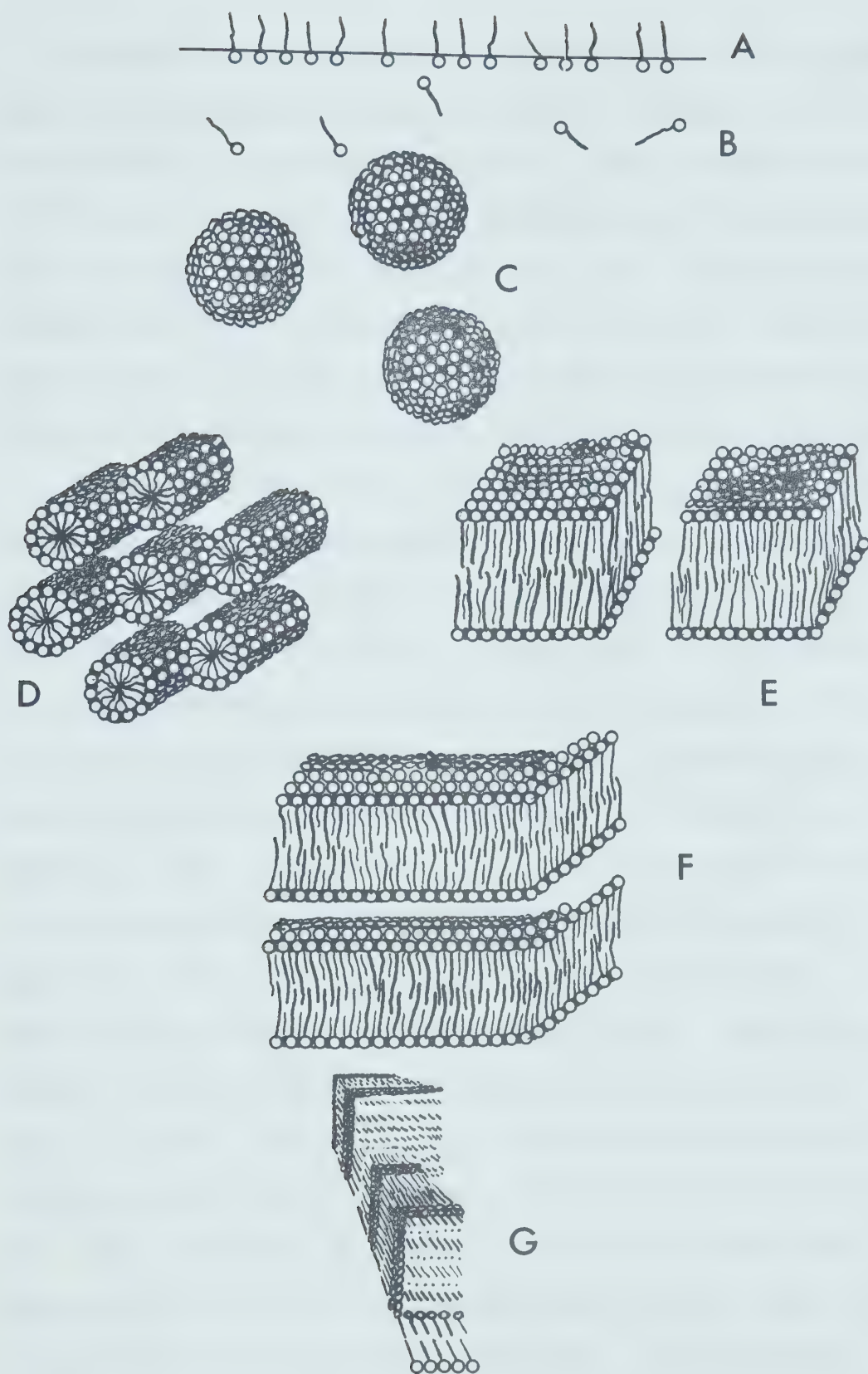
Liquid crystalline phases (50% w/v):

D - hexagonal variety,

E - cubic variety,

F - smectic or lamellar variety.

G - solid or coagel phase ($>90\%$ w/v).



Phospholipids essential to coagulation are supplied in vivo by platelets or damaged tissues. Platelet action in this respect is somewhat uncertain. The phospholipid may be on the platelet surface or be released by the platelet in the form of lipid-rich granules (91, 92). The platelet phospholipids may be supplied in the form of a lipoprotein complex (48). Tissue extracts, platelets, or dispersions of purified phospholipids are utilized for studies in vitro.

Specific single and mixed phospholipid suspensions exhibit a range of procoagulant to anticoagulant effects (93). Attempts to assign procoagulant activity to individual phospholipids or phospholipid combinations have produced contradictory results (93-96). Bangham (97) has correlated the clot promoting ability of phospholipids with their negative electrophoretic mobility, which in turn is related to the zeta potential and to the charge density on the phospholipid aggregates. This concept appeared to have resolved some of the previous conflict and has been supported by several investigators (95-98). However, more recent reports tend to contradict this generalization (66, 99, 100). Barton et al. (99) have shown that mixtures of phosphatidic acid (PA) and phosphatidyl choline (PC) are much less effective in a test coagulation system when compared with mixtures of phosphatidyl serine (PS) and PC at any given electrophoretic mobility. This finding suggests that the nature of phospholipid activity is more than a simple matter of particle charge. Wallach et al.

(101) have demonstrated that the colloidal state of the phospholipid particles as well as pH and ionic strength are additional variables.

Binding studies have shown that factor V is bound to phospholipid mixtures in the absence of Ca^{+2} and at low (0.005 M) Ca^{+2} concentrations (66, 86, 102). Factor Xa is also bound to phospholipid, but only in the presence of Ca^{+2} (64, 66, 102). Several investigators have shown that it is possible to form a prothrombin activator, consisting of a complex of factors V and Xa, Ca^{+2} and phospholipid, which can be sedimented by ultracentrifugation (66, 86, 103). The formation of a prothrombin activator has also been demonstrated using a gel filtration technique (102). In addition, prothrombin has been shown to bind to phospholipid mixtures in the presence of Ca^{+2} (22, 104). Thrombin is not bound in the presence or absence of calcium (104).

It has been suggested that prothrombin activation involves the binding of prothrombin to the lipid moiety of the prothrombin activator (factor Xa - Ca^{+2} - phospholipid - factor V) followed by conversion of prothrombin to thrombin by factor Xa and release of thrombin into the bulk aqueous phase (104, 105).

The object of this thesis project has been to investigate the nature of prothrombin-phospholipid binding. Previous studies have been restricted to equimolar mixtures of PS and PC. It seemed desirable to examine the nature of this interaction as a function of phospholipid composition

and, if possible, to correlate these results with the known effects of the particular phospholipids in coagulation assay systems.

At the outset of this project the available prothrombin preparation procedures yielded products which were either unstable or impure as judged by polyacrylamide disc electrophoresis. The preparation method developed to overcome these difficulties is similar to one published recently by Cox and Hanahan (12). The development of this method, together with a comparison of other recent procedures, has been included in a subsequent chapter.

CHAPTER II

MATERIALS AND METHODS

A. Materials

(NH₄)₂SO₄ (special enzyme grade, Lot V 4035) was obtained from Mann Research Laboratories (Orangeburg, N. Y.) and BaSO₄ from Merck (Rahway, N. J.). Diisopropyl fluorophosphate (DFP) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and prepared as a 1 M solution in isopropanol. Sephadex G-150 (Lot 860), Sephadex G-200 (Lot 8706), Sepharose 4B (Lot 8762), and Blue Dextran 2000 (Lot 2676), were obtained from Pharmacia (Montreal, Que.). The diethylaminoethyl (DEAE) cellulose used was a microgranular form (Whatman DE-32).

Prothrombin deficient substrate plasma was prepared according to Koller et al. (106) or was obtained as factor II and VII deficient plasma from Sigma (St. Louis, Mo.). Factor VII and X deficient plasma was also obtained from Sigma. Fibrinogen was prepared from bovine plasma by the method of Blombäck and Blombäck (107). Standard normal plasma was obtained from DADE (Miami, Fla.). Russell viper venom (Burroughs-Wellcome 'Stypven') was obtained from Warner-Chillcott (Scarborough, Ont.). Rabbit brain acetone powder for thromboplastin (Type 2, fine ground, without sodium citrate, desiccator dried) was obtained from Pel-Freez Biologicals (Rogers, Ark.) and was suspended in 0.14 M NaCl at a concentration of 3 g/100 ml and stored in

portions at -20°C . Brain lipid extract ("Cephalin") was prepared according to Bell and Alton (108) as a stock solution containing 120 μg P/ml and was made to a final concentration of 2 μg P/ml in Michaelis buffer, pH 7.35.

Phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were prepared from hen eggs by published methods (109, 110). Phosphatidyl serine (PS) was prepared from beef brain by a modification (111) of the method of Rouser et al. (112). Phosphatidic acid (PA) was prepared by the method of Kates (113), converted to the sodium form according to Ansell and Hawthorne (114) and stored in diethyl ether. Phosphatidyl choline (U-C-14) was obtained from Applied Science Laboratories (State College, Penn.).

Veronal-acetate buffer (pH 6.3 - 9.0) was prepared by dissolving sodium acetate trihydrate (19.528 g), sodium diethyl barbiturate (29.528 g) and 34 g of NaCl in 4 litres of water. The pH of the solution was adjusted as required with 1 N HCl or 1 N NaOH and the volume made up to 5.4 litres with water.

All other solvents and chemicals used were of reagent grade or better. Water used was distilled and deionized.

B. Methods

1. Phosphorus determinations

Phospholipid phosphorus was determined according to the method of King (115). Total phospholipid was determined using a conversion factor of 25 for PS, PC, PE and 21 for

PA, by assuming molecular weights of 775 for PS, PC and PE, and 625 for PA.

2. Protein determinations

Protein concentrations were determined either by absorbance of solutions at 280 nm or by the method of Lowry et al. (116). A_{280} readings were corrected for Rayleigh light scattering by the method of Shapiro and Waugh (117). $E_{1\text{ cm}}^{1\%}$ for prothrombin was taken as 14.0 (12).

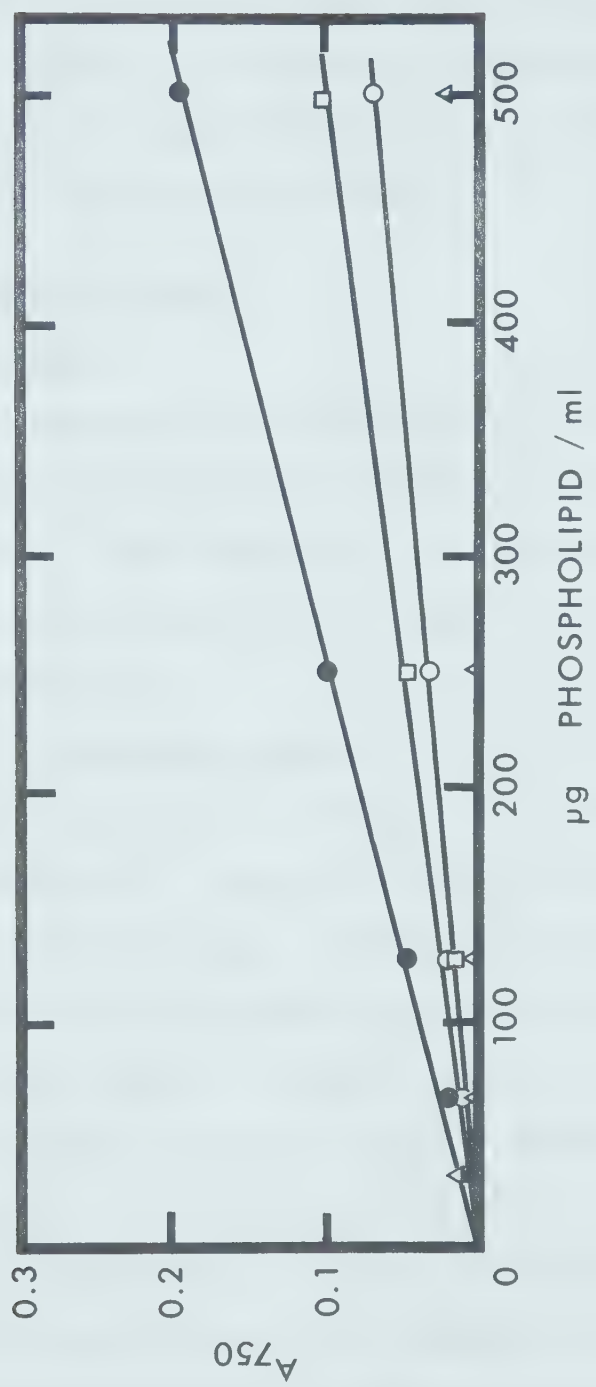
It was found that PS and PE interfered in protein determinations using the method described by Lowry et al. resulting in positive A_{750} readings in the absence of protein (Fig. 6). These effects were routinely corrected using a program written for an Olivetti-Underwood Programma 101 calculator.

3. Preparation of ion exchange cellulose

DEAE-cellulose was prepared according to directions supplied with the material (Whatman Information Leaflet IL-2). This procedure involved stirring 100 g (dry weight) of ion exchange cellulose into 1.5 litres of 0.5 N HCl and leaving for 1 hour. The supernatant was then decanted and the slurry washed until the pH of the supernatant reached 4. The procedure was then repeated with 0.5 N NaOH and washing carried out until the pH of the supernatant reached 8. Equilibration was carried out by washing the gel with 0.05 M phosphate buffer, pH 7.2, until the supernatant pH

Figure 6. Interference of phospholipid dispersions in protein determinations.

Sonicated phospholipids were diluted in veronal-acetate buffer, pH 9.0, containing 0.010 M CaCl_2 and assayed by the protein determination method of Lowry et al. (Ref. 116). Δ — Δ , PA; \square — \square , PE:PC (50/50 w/w); \circ — \circ , PS:PC (30/70 w/w); \bullet — \bullet , PS.



remained at 7.2.

After use, the ion exchange cellulose was regenerated by repeating the entire procedure. DEAE-cellulose was stored at 4° C in water containing 0.02% sodium azide as a bacteriostatic agent. Equilibration was carried out prior to use of the ion exchange cellulose.

4. Coagulation assays

a. Thrombin

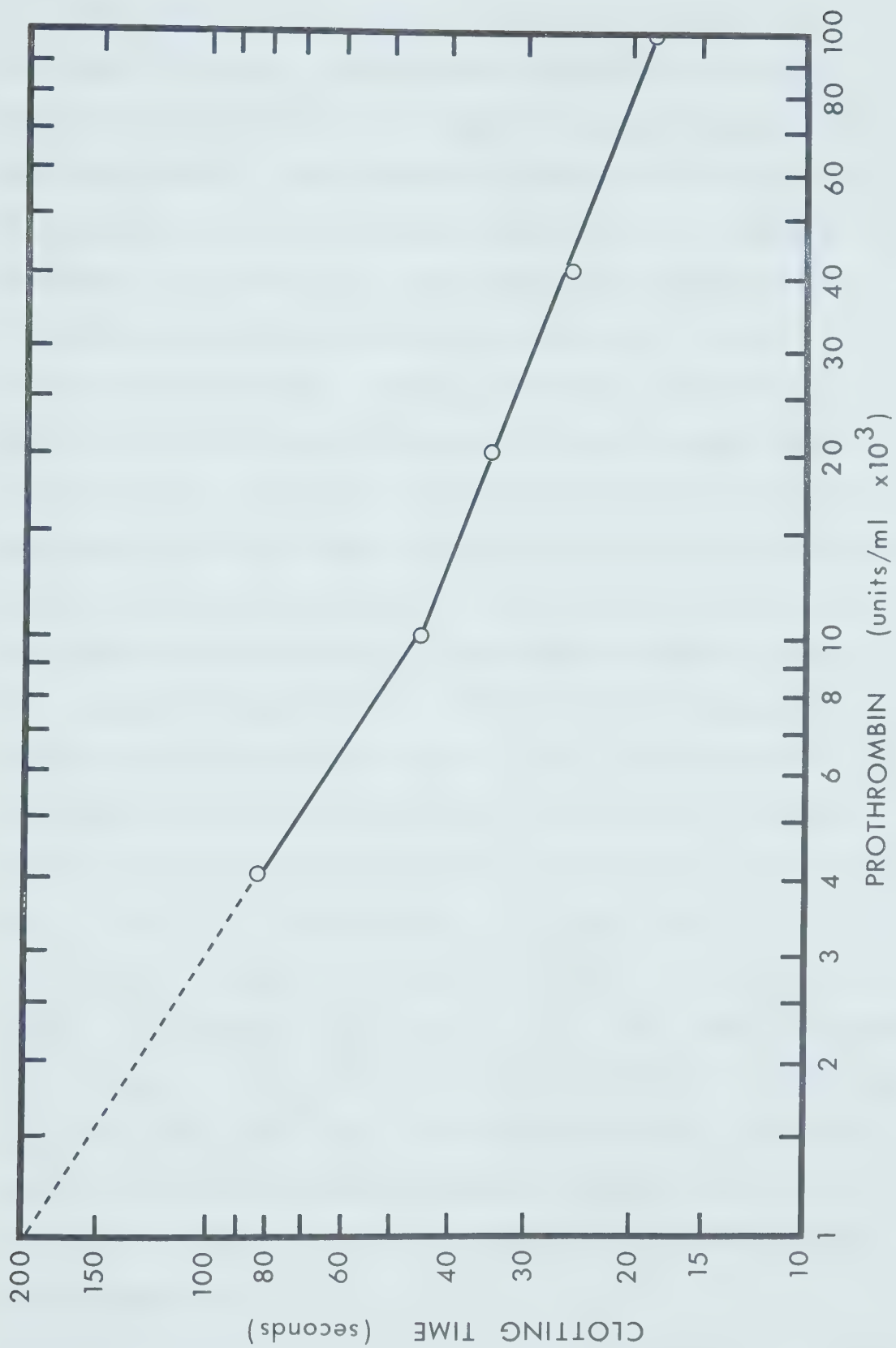
Thrombin was assayed by incubating 0.2 ml of test sample with 0.2 ml of fibrinogen solution (2 mg/ml) at pH 7.35 and 37° C. Each result was recorded as the time required for the first trace of a fibrin clot formation.

b. Prothrombin

i. one-stage assays

Prothrombin was routinely assayed by either of two "one-stage" procedures. These were the tissue thromboplastin assay of Koller et al. (106), used in conjunction with prothrombin deficient substrate plasma (106), and the Russell viper venom (RVV) - cephalin assay of Hjört et al. (118) which was used in conjunction with commercially obtained factor II and VII deficient plasma. Both assay systems were calibrated by measuring the clotting times obtained with various dilutions of standard plasma and plotting these against units of activity on log-log graph paper. A typical calibration curve is shown in Fig. 7.

Figure 7. Calibration curve for one-stage prothrombin assay. One unit of prothrombin is defined as that fraction present in 1 ml of normal, pooled human plasma.



The assay of Koller et al. utilizes the extrinsic coagulation pathway. Prothrombin deficient plasma was prepared by mixing 1:1 (v/v) BaSO₄ adsorbed bovine plasma and human serum. The adsorbed plasma is free of factors II (prothrombin), VII, IX and X and serves as a source of fibrinogen and factor V. Human serum was obtained by mixing brain lipid extract with fresh blood. This procedure removes most of the prothrombin and leaves sufficient quantities of factors VII and X since these are only partially consumed. Storage of the clotted blood overnight at 37° C allows time for inactivation of thrombin by heat denaturation and by an inhibitor. Tissue extract was supplied in the form of rabbit brain acetone powder.

Assays were carried out at 37° C by mixing equal volumes (0.1 ml) of test sample, brain tissue extract, prothrombin deficient plasma and 0.025 M CaCl₂ solution. Clotting times were measured from the time of addition of CaCl₂ to the first appearance of a fibrin clot.

The RVV - cephalin assay is based on the activation of factor X by Russell viper venom (119). The assay procedure was the same as that described for the assay of Koller et al. except that the brain tissue extract was a "cephalin" solution containing a 200,000 fold dilution of venom and the deficient plasma was deficient in factor VII as well as prothrombin.

ii. two-stage assay

The two-stage assay procedure of Ware and Seegers (120) was used in one case (page 41). This assay also utilizes the extrinsic activation pathway. It differs from one-stage assays in that prothrombin is converted to thrombin in an incubation mixture free of fibrinogen. Aliquots of the incubation mixture are then assayed for thrombin activity by measuring the time required to clot a standard fibrinogen solution. The two-stage assay tends to be somewhat more reliable than a one-stage assay in that maximum formation of thrombin from the available prothrombin is achieved. In a one-stage assay a clot can be formed when relatively little of the available prothrombin has been activated.

The two-stage assay is cumbersome and time consuming. For this reason prothrombin assays were routinely carried out using one-stage procedures.

c. Factor X

Factor X was assayed by the method of Bachmann et al. (121). The assay procedure is identical to the RVV - cephalin assay for prothrombin except that the substrate plasma is deficient in factors VII and X.

5. Preparation of lipid dispersions

All phospholipids used in gel filtration and precipitation experiments were prepared at concentrations of 5 mg/ml in distilled, deionized water. pH adjustments were

made with μ l amounts of 1 N KOH or 1 N HCl. Uniformly labelled ^{14}C -PC was added to each sample for use as a tracer to measure phospholipid concentration. The final concentration of added ^{14}C -PC was never greater than 0.5 $\mu\text{g/ml}$. Final specific activities were of the order of 30 cpm/ μg of phospholipid.

Solvent was removed from measured volumes of stock phospholipid solutions in either chloroform or diethyl ether by streaming nitrogen over the sample. Further drying was carried out by placing samples in a vacuum oven at room temperature overnight. The phospholipids were then dispersed in water by hand-shaking. After adjusting the pH, the dispersions were sonicated using a Raytheon DF 101 10 kHz sonic oscillator at maximum power setting. Total sonication time was from 15 to 45 minutes as required for samples to become transparent. Sonication was interrupted every 5-10 minutes to check and adjust pH. A circulating coolant kept sample temperatures below 10°C during sonication.

6. Radioactivity assays

^{14}C -levels were determined with a Nuclear Chicago MK 1 scintillation counter. Scintillation vials each contained 10 ml of scintillation fluid which was prepared according to Bray (122). 0.1 ml aliquots of test samples were transferred to vials for counting. Counting was carried out at 10°C .

The ^{14}C radioactivity of stock solutions of sonicated phospholipids was determined from the average of duplicate 10 minute counts. The specific activities of these solutions were then calculated in terms of μg of total phospholipid/CPM. The count deviations, at levels counted, were less than 1% at the 90% confidence level.

Fractions assayed in Chapter IV were counted for 4 minutes each. At this time interval the standard deviation of counts was less than 3% for peak fractions and 8% at background level. Fractions were closely spaced so that the data were readily averaged by drawing elution profiles.

Samples assayed in Chapter V were counted for 20 minutes each. Standard deviations on all samples were less than 3%.

7. Amino acid analyses

Amino acid analyses of prothrombin were carried out with a Beckman Model 120B amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110°C for 24 (duplicates), 48 and 72 hours (single samples) in sealed, evacuated tubes. The numbers of serine and threonine residues were obtained by extrapolation to zero time. Valine and isoleucine values were determined from the 72 hour hydrolysate data. Tryptophan was determined spectrophotometrically as described by Goodwin and Morton (123).

8. Electrophoresis

Analytical polyacrylamide disc electrophoresis was carried out in Tris-HCl buffer, pH 8.6, according to the method of Ornstein (124) and Davis (125) as described in a Canalco Manual (125).

Gels were scanned at 600 nm using a Gilford Model 240 spectrophotometer fitted with a gel scanning attachment. A 0.05 x 2.36 mm slit was used.

Polyacrylamide gels were also used to estimate the molecular weight of prothrombin using a method described by Maizel (127). After treatment with 1% 2-mercaptoethanol and 2% SDS protein samples were subjected to electrophoresis on 5% acrylamide gels in 0.1 M phosphate buffer, pH 7.2 with 0.1% SDS. Calculations were carried out by the method of Weber and Osborn (128). Proteins used for calibration were aldolase, Blue Dextran 2000, bovine serum albumin (BSA), chymotrypsinogen, cytochrome c and ovalbumin.

9. Molecular weight estimation by gel filtration

The method of Andrews' (129) was used to obtain an estimate of the molecular weight of prothrombin by gel filtration. A Pharmacia K 9/60 column (0.9 cm diameter x 60 cm height) packed with Sephadex G-200 was used. Sample size was 0.40 ml. Samples were eluted at 4^o C using upward flow, with 0.15 M Tris-HCl buffer, pH 7.4, containing 0.010 M KCl. Aldolase, BSA, chymotrypsinogen, cytochrome c and ovalbumin were used as calibration proteins.

10. Ultracentrifuge studies

These studies were carried out at 20° C using a Beckman Model E ultracentrifuge. Sedimentation coefficients were obtained using Schlieren optics. Calculations were carried out using an APL / 360 program (see Appendix). Rayleigh interference optics were used for the determination of diffusion coefficients according to a procedure described by Chervenka (130). Molecular weights were determined by the high speed equilibrium method of Yphantis (131) and the conventional, low speed, equilibrium method as outlined by Chervenka (132). Molecular weights by the low speed equilibrium method were calculated as a function of concentration across the cell using an APL / 360 program developed by William Wolodko of this Department. All linear graphs were determined by a least-squares fit. The partial specific volume of prothrombin was taken as 0.70 ml/g (12, 19).

11. Difference spectroscopy

Ultraviolet spectra were obtained using a Cary 15 spectrophotometer scanning a wavelength range of 340 to 230 nm. Temperatures were maintained at 25° C with a circulating water bath.

CHAPTER III

PREPARATION AND CHARACTERIZATION OF PROTHROMBIN

A. Introduction

Prothrombin purification procedures have evolved from techniques developed by Mellanby (133) and Fuchs (134) in which prothrombin was recovered from plasma by isoelectric precipitation, adsorbed onto $\text{Mg}(\text{OH})_2$ and then eluted and further processed. These procedures were subsequently refined by Seegers (135) and Magnusson (20). Additional procedures involving adsorption onto other salts such as barium citrate (15, 136, 137), barium sulphate (12, 138) and various clays (139) have been presented. Among the contaminants of these preparations are the coagulation factors VII, IX and X which have adsorption characteristics similar to prothrombin (140). The concentrations of some of these factors may be reduced by filtration through Seitz filters (138), by ion exchange chromatography (82) and by an isoelectric focussing procedure (141).

At the outset of this project, prothrombin preparation procedures yielded products which were heterogeneous by the technique of polyacrylamide disc electrophoresis. The objective was to develop a method for the preparation of a prothrombin product which was a single component by disc electrophoresis and contained negligible quantities of other coagulation factors. At least two subsequent

publications (12, 14) describe methods which meet these criteria.

An outline of the preparation procedure and product characterization follows. A comparison between this preparation and others has been included.

B. Preliminary Preparation Attempts

Initial prothrombin preparative work was carried out using the $\text{Mg}(\text{OH})_2$ adsorption technique described by Magnusson (20). Investigation of the adsorption effects of $\text{Mg}(\text{OH})_2$ indicated that removal of 90% of the prothrombin activity from a crude prothrombin solution (obtained from isoelectric precipitation of plasma) required four successive adsorptions using optimal concentrations of $\text{Mg}(\text{OH})_2$. BaSO_4 was found to adsorb 100% of the prothrombin activity directly from plasma and was therefore used directly with plasma, eliminating the preceding isoelectric precipitation step. Subsequent chromatography of the BaSO_4 eluate on Sephadex G-200 followed by DEAE-cellulose chromatography yielded a prothrombin product which had a prothrombin activity of 1 unit/mg and appeared to be a single peak in the analytical ultracentrifuge.

Attempts to scale up this procedure from 2 litres of plasma to 10 or 20 litres led to considerable difficulties with prothrombin degradation and loss of activity. This appeared to be due in part to the longer times required in the preparation. These difficulties seemed to be resolved

by using 10^{-4} M DFP in all purification steps subsequent to BaSO_4 adsorption.

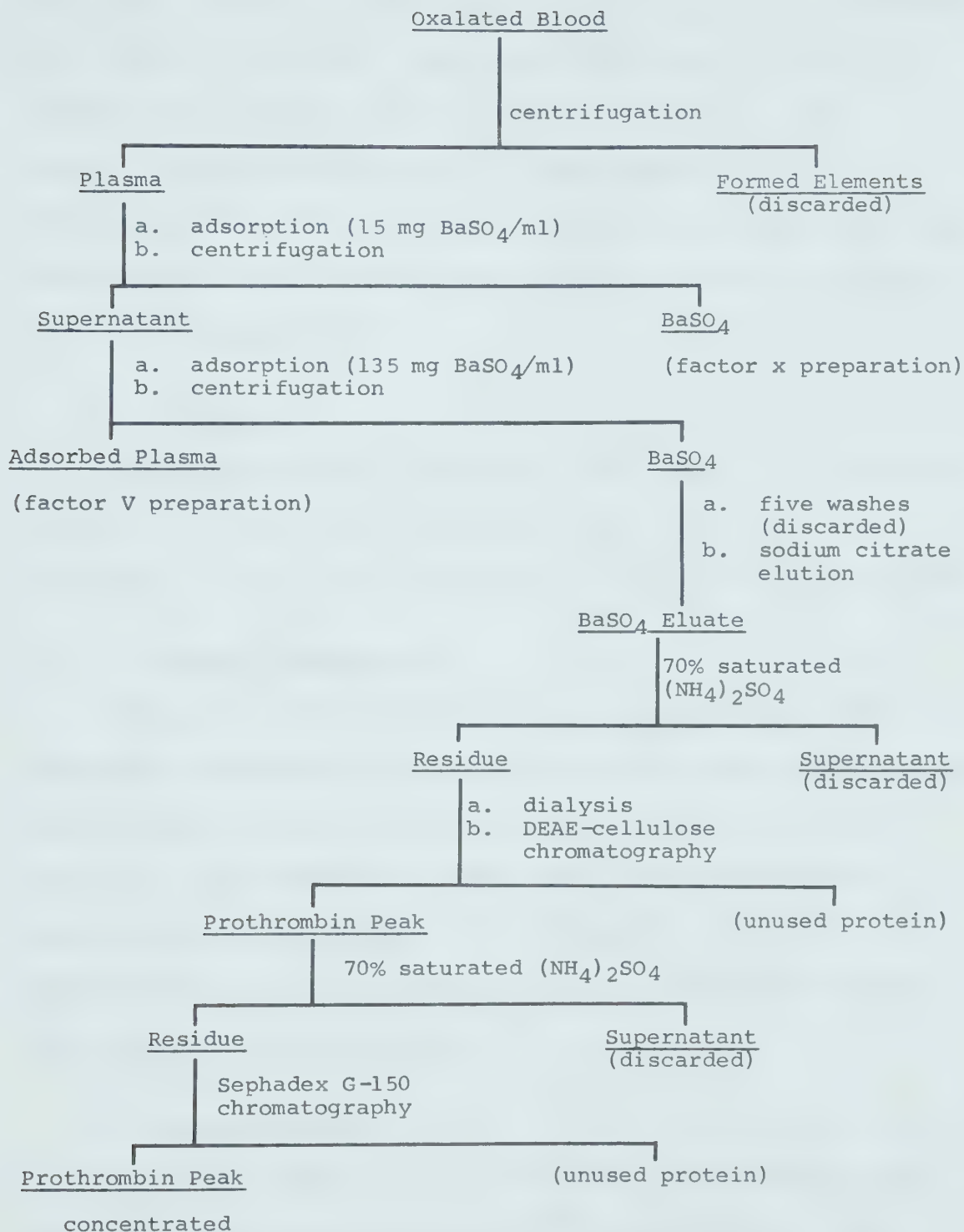
Other attempts to prepare prothrombin were from two different approaches. The method of Tishkoff et al. (15) involved the preparation of crystals of barium citrate, prothrombin and factors VII, IX and X followed by purification of the dissolved crystals. Problems were encountered in crystallization. For this reason and the fact that the final product reported was not significantly better than that obtained by previous methods, this approach was abandoned. Commercial prothrombin (Mann Chemicals Lot # T4443), obtained for purposes of further purification, was found to contain no prothrombin activity.

The final purification procedure is shown in the flow chart in Fig. 8. The procedure provides, as by-products, starting materials for the preparation of factors V and X.

C. Preparation Method

Bovine blood was used as a source of prothrombin. Unless otherwise noted, most of the procedures were carried out at room temperature. Except in the case noted in Step 5 below, centrifugation was carried out with an International PR-6 centrifuge fitted with a 276 rotor. All solutions from the BaSO_4 washes onward contained 10^{-4} M DFP.

Figure 8. Flow chart of the prothrombin preparation procedure.



1. Blood collection

Blood was obtained at the slaughter house by severing the great vessels of the neck of cattle previously stunned by a blow on the head. Blood was collected in 15 litre batches in a vessel containing 1.5 litres of 0.1 M sodium oxalate anticoagulant. The blood was stirred immediately to ensure adequate mixing of the anticoagulant. The blood was then transported directly to the laboratory without refrigeration.

2. Plasma

The blood was centrifuged at 2500 rpm for 20 minutes. Plasma was removed by gentle aspiration and the blood cells discarded. Plasma was processed in 10 to 15 litre batches.

3. BaSO₄ adsorption

BaSO₄ was added to the plasma in two stages. The first adsorption required 15 g BaSO₄/litre of plasma. The mixture was stirred for 30 minutes with an electrically driven propeller-type apparatus. The BaSO₄ was recovered by centrifugation (15 minutes at 2200 rpm). A second adsorption using 135 g BaSO₄/litre of plasma was carried out using the same procedure.

4. BaSO₄ washes

The BaSO₄ cakes obtained from the second adsorption step were washed with cold (4-10° C) 0.45% NaCl. This operation was performed by placing the BaSO₄ in a Waring

blender and adding approximately 100 ml of saline solution per 100 g dry weight of BaSO_4 . Blending time was 3 minutes. The BaSO_4 was recovered by centrifugation (15 minutes at 2200 rpm). This step was repeated five times.

5. Elution of prothrombin from BaSO_4

The washed BaSO_4 was then blended with cold (4°C) 0.14 M sodium citrate-citric acid buffer, pH 5.8, using a Waring blender. The volume of buffer used was one tenth of the plasma volume. After blending, the mixture was stirred for 30 minutes at room temperature. The BaSO_4 was removed by centrifugation (15 minutes at 2200 rpm). The supernatant was then concentrated by precipitation with 70% saturated $(\text{NH}_4)_2\text{SO}_4$ (430 g/litre). The precipitate was recovered by centrifugation for 2 hours at 3300 rpm and redissolved in a minimum volume of 0.01 M phosphate buffer, pH 7.2. The solution was then dialysed against 60 litres of 0.01 M phosphate buffer, pH 7.2, for 12 to 14 hours. A small amount of precipitate which formed during dialysis was removed by centrifugation at $3000 \times g$ for 20 minutes (Sorvall RC-2B).

6. DEAE-cellulose chromatography

Chromatography on DEAE-cellulose was carried out at 4°C . A slurry of DEAE-cellulose (100 g dry weight), previously equilibrated with 0.05 M phosphate buffer, pH 7.2, was poured into a 6 cm diameter glass column just prior to

chromatography. After loading the column, the first solvent used was 0.075 M phosphate buffer, pH 7.2. Flow rates were maintained between 3 and 5 ml/minute by adjustment of the hydrostatic pressure head. This eluant was used until the A_{280} of the eluate decreased below 0.1. Additional protein was removed with 0.1 M phosphate buffer, pH 7.45. These initial eluate fractions were discarded. The eluting solvent was then changed to a linear gradient of 0.1 M to 0.175 M phosphate buffer, pH 7.45. The prothrombin-containing fractions of this eluate were pooled and concentrated by adding $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation (430 g/litre). The precipitate was recovered by centrifugation (2 hours at 3300 rpm) and redissolved in a minimum volume (15 to 20 ml) of veronal-acetate buffer, pH 6.5, for application to a Sephadex gel column.

7. Sephadex G-150 chromatography

Columns for gel filtration were made of plexiglass tubing, measuring 4.8 cm diameter by 100 cm height. They were filled with Sephadex G-150 and equilibrated at 4° C with veronal-acetate buffer, pH 6.5, containing 10^{-3} M EDTA. Protein was eluted with the same buffer. Flow rates were 25 to 30 ml/hour using an upward flow system. The prothrombin-containing fractions were pooled and concentrated with an Amicon Diaflo ultrafiltration cell fitted with a UM-10 filter. The sample was usually concentrated to 5 mg/ml and stored at - 20° C.

D. Results and Discussion

The initial stages of this preparation procedure, including the anticoagulant, the use of BaSO_4 and the sodium citrate elution, are similar to the initial stages of factor X preparation methods described by Duckert et al. (142) and Papahadjopoulos et al. (143). The elution scheme for the DEAE-cellulose fractionation is similar to that reported by Seegers and Landaburu (144). The use of DFP in prothrombin preparations has been reported previously (12) and has proven essential in preventing excessive degradation of prothrombin. DFP has also been used in the preparation of factor X (145). The effect of DFP is presumably to inhibit some serine proteinases responsible for the degradation of prothrombin. Among the possible enzymes implicated are factor Xa and thrombin. A number of the stages of the preparation were examined with the view of optimizing recovery and purity of prothrombin.

1. BaSO_4 adsorption

The amount of BaSO_4 required was found to differ with various commercial preparations of the salt as shown in Fig. 9. Complete removal of prothrombin was desirable both for this preparation and for the preparation of factor V from the adsorbed plasma.

The first step of the two-stage BaSO_4 adsorption procedure considerably reduced the plasma factor X concentration (Table II). Prothrombin was also removed

Figure 9. Adsorption of prothrombin from bovine plasma by BaSO_4 .

BaSO_4 was added to aliquots of plasma in amounts ranging from 0 to 250 mg/ml of plasma. Mixtures were stirred for 30 min. at 25°C and then centrifuged for 30 min. at 1500 x g. Supernatants were decanted and assayed for prothrombin activity. Δ — Δ , BaSO_4 (Fisher Chemicals, Cat. No. B-68, Lot 720595). \square — \square , BaSO_4 (British Drug Houses (Canada) Ltd., Lot 4279A). \bullet — \bullet , BaSO_4 (Mallinckrodt Chemical Works Ltd., U.S.P. for X-ray diagnosis, Cat. No. 3808).

TABLE II

ADSORPTION OF PROTHROMBIN AND FACTOR X
BY BaSO₄

Amount of BaSO ₄ Added (g/litre of plasma)	Level of Activity Remaining in adsorbed plasma			
	Prothrombin		Factor X	
	unit/ml	% of original	unit/ml	% of original
0	0.45	100	0.74	100
10	0.35	78	0.20	27
20	0.28	62	0.029	4

by this procedure, although the amount removed was proportionately less than the amount of factor X removed. This procedure provided material for the preparation of factor X and at the same time reduced the level of factor X in the prothrombin preparation. This was desirable since in its active form, factor X hydrolyses prothrombin to thrombin.

2. BaSO₄ washing

The washing procedure removed a considerable amount of protein remaining in the cake of BaSO₄. The half-isotonic saline also lyses any remaining blood cells. It was found that five washings reduced the absorbance of the final wash to less than 0.01 A₂₈₀ units. No prothrombin was eluted by this procedure.

3. Elution of prothrombin from BaSO₄

0.14 M citrate buffer, pH 5.8, was found to be optimal for the elution of prothrombin activity as shown in Table III. Recovery of prothrombin activity at this stage, as measured by the one stage assay, was 10% to 15%. Examination of the recovery using a two-stage assay revealed a recovery of approximately 70%. Voss (140) has reported recoveries of 70% to 80% using a similar procedure. Total A₂₈₀ units at this stage was 3,000 to 3,500 for 10 litres of plasma.

TABLE III

ELUTION OF ADSORBED PROTHROMBIN
FROM BaSO₄ WITH SODIUM CITRATE

Molarity of Citrate Eluant	Amount of Prothrombin Activity in Eluate (units)
0.105	1.56
0.14	2.04
0.175	1.63
0.21	1.46
0.245	1.39

Prothrombin was adsorbed to BaSO₄ from bovine plasma (150 mg BaSO₄/ml plasma). The BaSO₄ was removed by centrifugation (1500 x g for 30 minutes at 0° C) and washed three times in 0.45% NaCl. Equal portions of BaSO₄ were then suspended in varying concentrations of citrate buffer, pH 5.8 (0.15 x volume of plasma) and stirred for 1 hour at 4° C. The mixtures were then centrifuged (2000 x g for 30 minutes at 4° C) and the supernatants assayed for prothrombin activity.

4. Chromatographic steps

Typical elution profiles obtained by ion-exchange and gel filtration chromatography are shown in Fig. 10 and Fig. 11 respectively. The prothrombin solution obtained from DEAE-cellulose chromatography was found to contain one major component together with a small amount of more rapidly sedimenting components when examined in the analytical ultracentrifuge (Fig. 12a). When concentrated to 14 mg/ml (Fig. 12b), the main component had an S_{obs} value of 4.7 S. A second component, apparent when the sample was concentrated, had an S_{obs} value of approximately 11 S. The gradient elution scheme was a modification of the step-wise elution used by Seegers and Landaburu (144). Gel filtration separated the 11 S contaminant from prothrombin (Fig. 11).

5. Prothrombin yield

The overall yield of prothrombin by this method was 150 to 180 mg from 10 litres of plasma. Based on a bovine blood prothrombin content of 0.007% w/v (135) this represents a protein recovery of 10% to 15%.

E. Characterization of Prothrombin

In view of previous work on the preparation and characterization of prothrombin (12, 14, 15, 19, 20, 78, 135), a number of physico-chemical procedures were carried out to provide a comparative characterization of the prothrombin prepared by this method.

Figure 10. DEAE-cellulose chromatography of prothrombin eluted from BaSO_4 . Column dimensions were 25 cm (length) by 6 cm (diameter). Total A_{280} units of protein applied to the column was 9200. Flow rates were maintained between 3 and 5 ml/minute. Prothrombin was eluted from the column with a linear gradient of 0.1 M to 0.175 M phosphate buffer, pH 7.45, containing 10^{-4} M DFP. Two initial fractions were eluted with 0.075 M phosphate buffer, pH 7.2, and 0.1 M phosphate buffer, pH 7.45. The total A_{280} units of protein eluted with the two initial solvents were 2100 and 190 respectively. $\square \text{---} \square$ represents A_{280} . $\text{O} \text{---} \text{O}$ represents prothrombin activity.

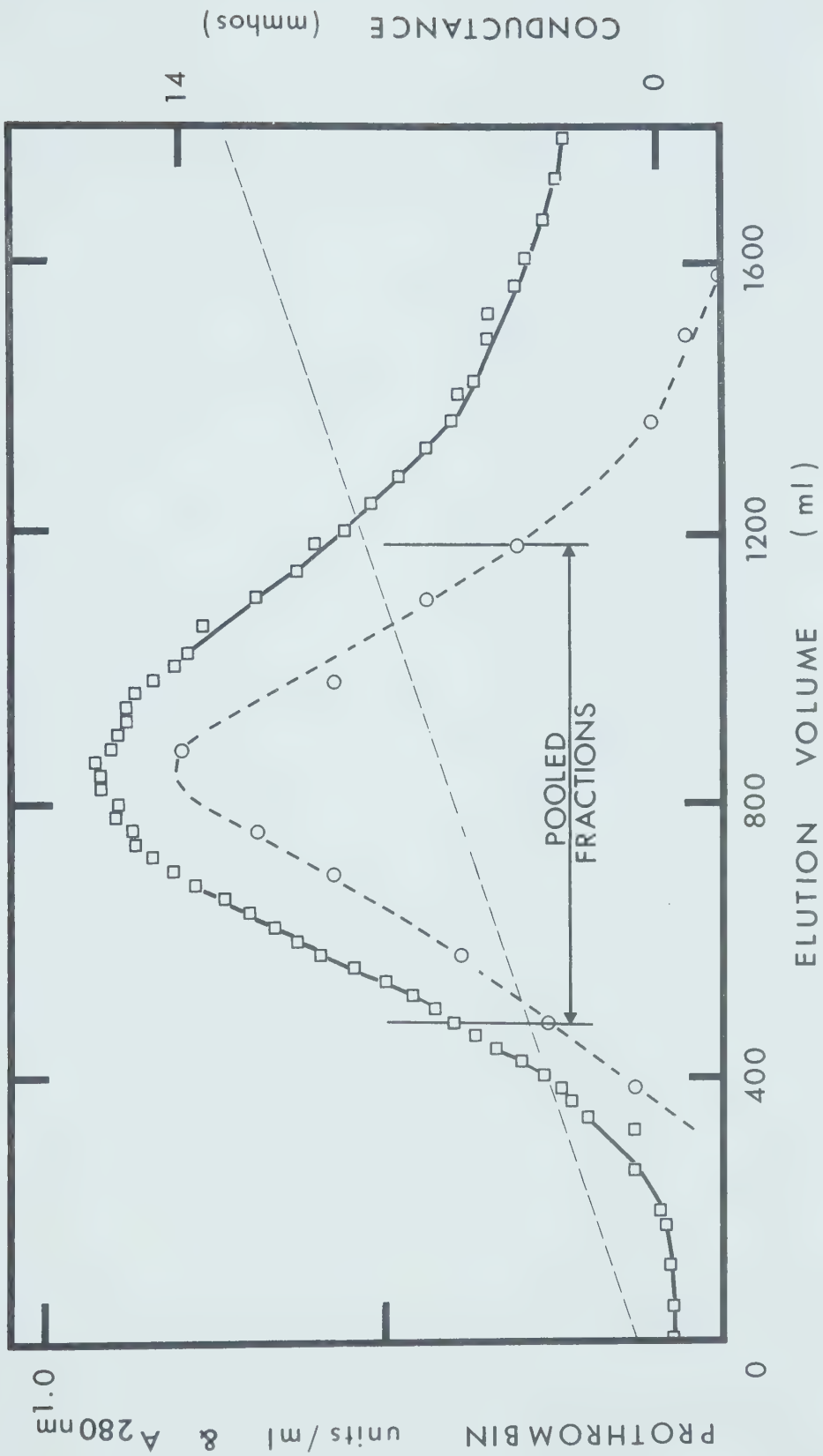


Figure 11. Chromatography on Sephadex G-150 of prothrombin-containing peak from DEAE-cellulose column. Dimensions of column were 100 cm (length) by 4.8 cm (diameter). Eluting solvent was veronal-acetate buffer, pH 6.5, containing 10^{-4} M DFP and 10^{-3} M EDTA. Sample volume was 19 ml. Total A_{280} units of protein applied to the column was 390. \square — \square represents A_{280} . O---O represents prothrombin activity.

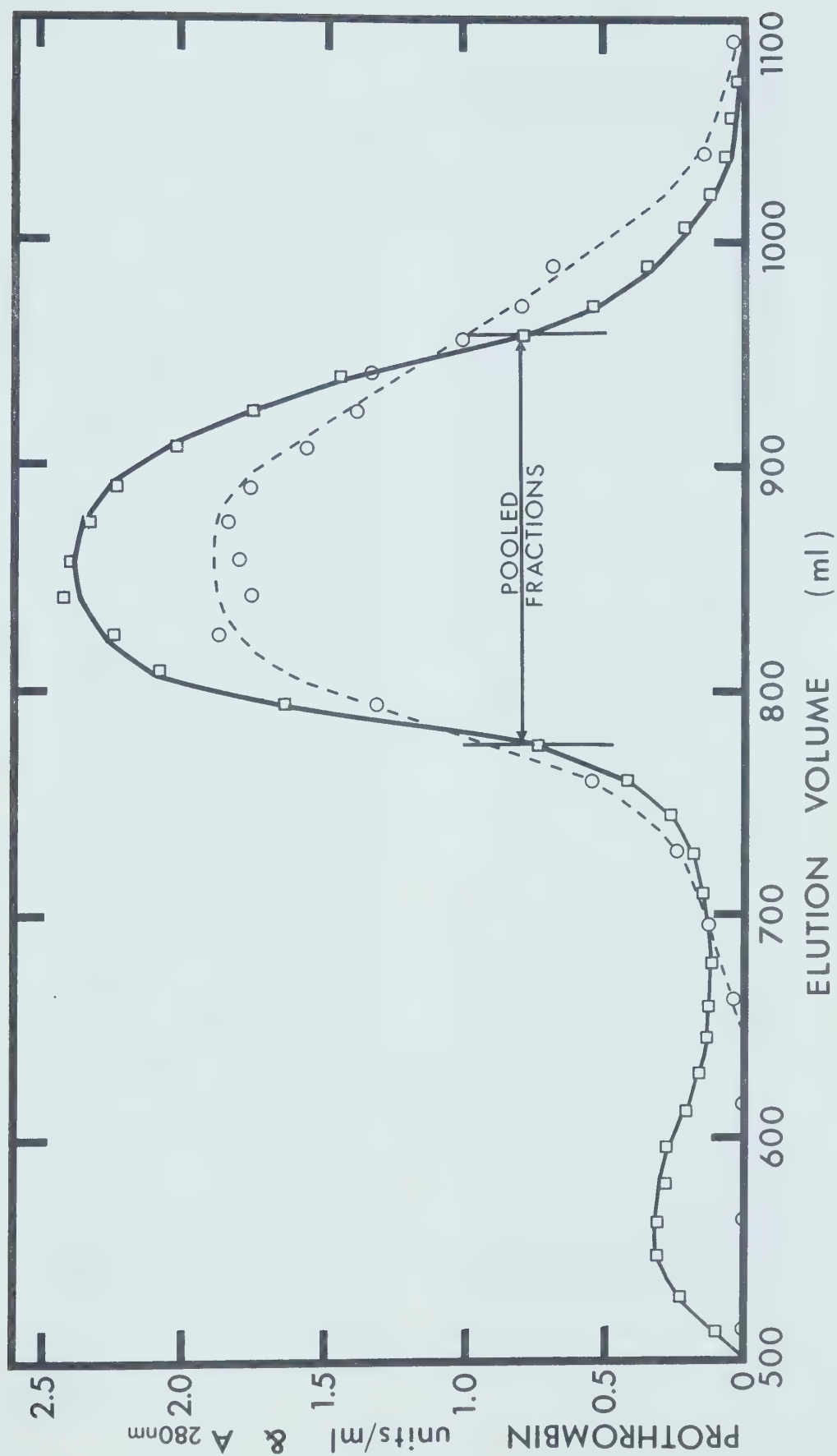
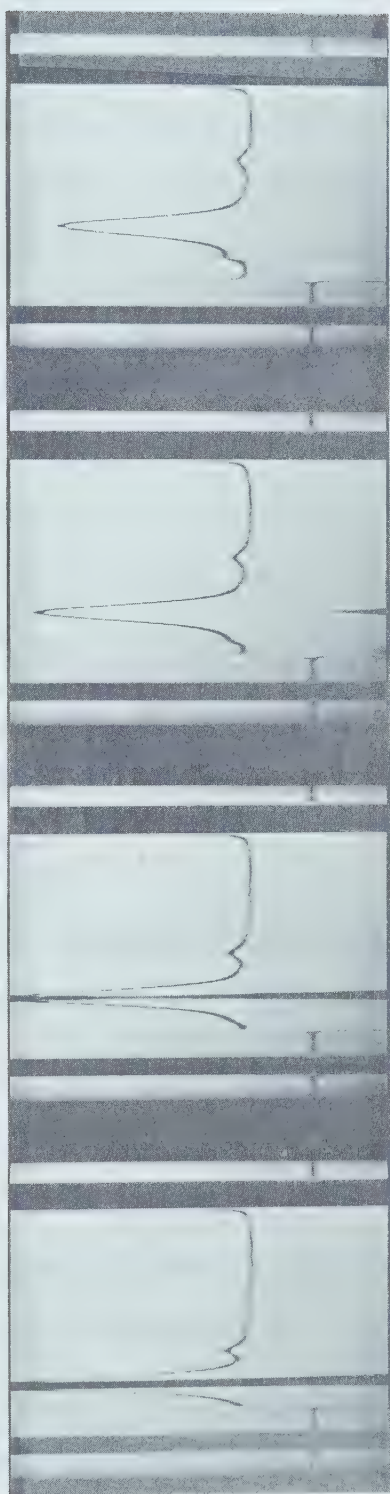


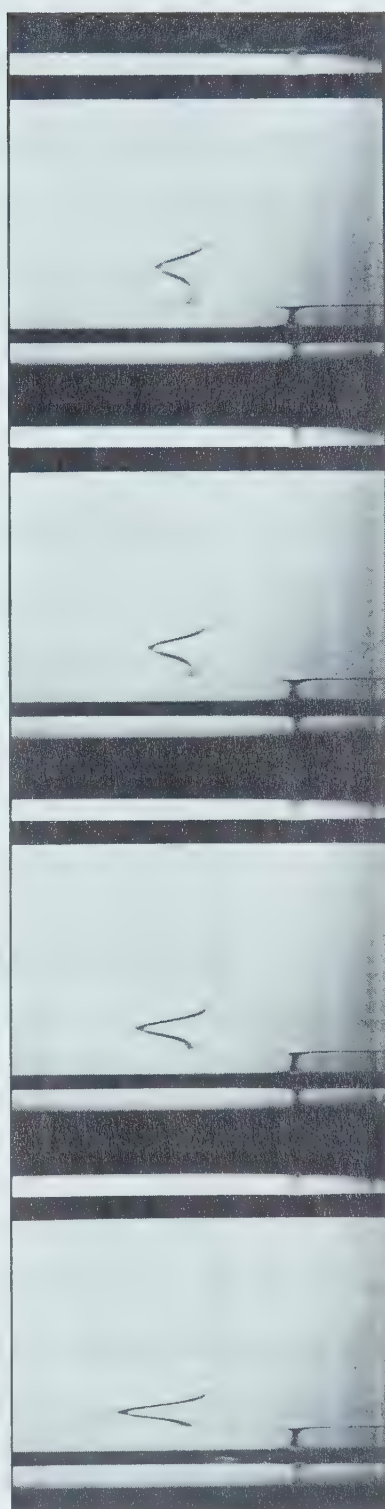
Figure 12. Schlieren patterns obtained by ultracentrifugation of prothrombin-containing peak after DEAE-cellulose column chromatography. A Beckman Model E ultracentrifuge was used. Rotor speed was 60,000 rpm; temperature 20° C. Photographs were taken at 8 minute intervals.

Upper photo (a); a peak fraction from DEAE-cellulose column. Sample concentrated to A_{280} of 4.2 in 0.175 M phosphate buffer, pH 7.45.

Lower photo (b); concentrated sample from pooled prothrombin containing fractions. Sample concentration 14 mg/ml in veronal-acetate buffer, pH 6.5.



a



b

1. Polyacrylamide disc electrophoresis

A polyacrylamide gel pattern obtained from electrophoresis of a 50 μ g sample of prothrombin is shown in Fig. 13. Only a single component is apparent on visual examination or by spectrophotometric scanning at 600 nm. The band observed appeared somewhat diffuse. Similar results have been reported by Ingwall and Scheraga (14) and Cox and Hanahan (12).

The molecular weight of prothrombin, estimated by electrophoresis on 5% polyacrylamide gels, was 84,000 \pm 3,000 (Fig. 14). After treatment with 1% 2-mercaptoethanol and 2% SDS the protein still appeared as a single component. This offers further support for the supposition that prothrombin consists of a single polypeptide chain (13-15).

2. Molecular weight by gel filtration

The apparent molecular weight of prothrombin, by this method, was found to be 110,000 (Fig. 15). When compared with the results obtained from equilibrium ultracentrifugation data (below), this value suggests molecular asymmetry and/or aggregation. Tishkoff et al. (15) have reported gel filtration studies which do suggest that prothrombin undergoes rapid, reversible association in buffers of ionic strength below 0.6. Cox and Hanahan (12) have carried out similar concentration dependence studies using Sephadex G-150 and have reported an extrapolated,

Figure 13. A polyacrylamide disc electrophoresis gel of purified prothrombin. Sample size was 50 μ g. Gels were stained with amido schwarz. Migration was from top to bottom (toward the anode).



Figure 14. Estimation of the molecular weight of prothrombin by polyacrylamide disc electrophoresis. Prothrombin and calibration proteins were treated with 1% 2-mercaptoethanol and 2% SDS prior to electrophoresis. Samples were subjected to electrophoresis on 5% acrylamide gels in 0.1 M phosphate buffer, pH 7.2, with 0.1% SDS. Relative migration was calculated by the method of Weber and Osborn (Reference 128). The relative migration of prothrombin is indicated by a vertical arrow.

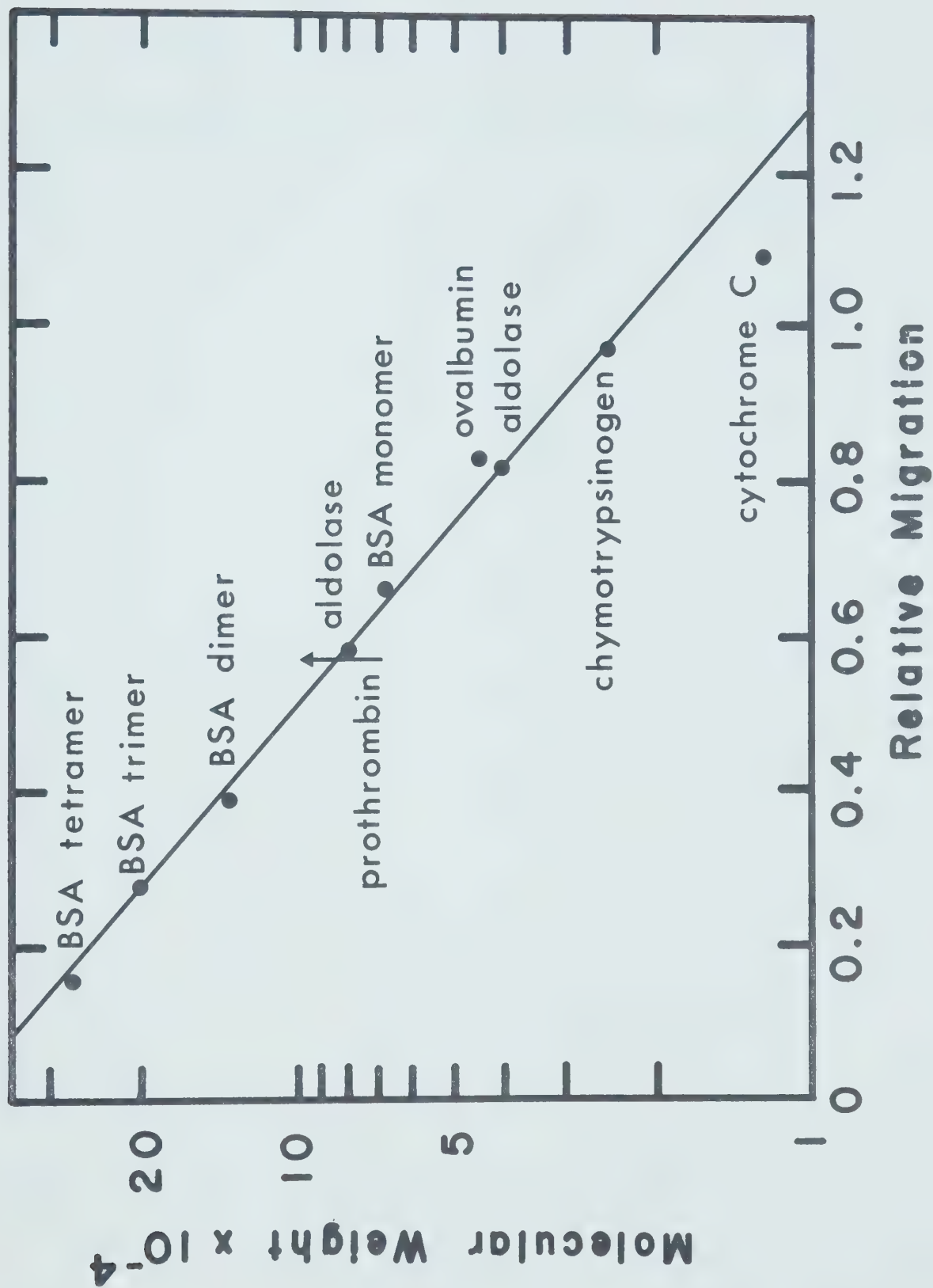
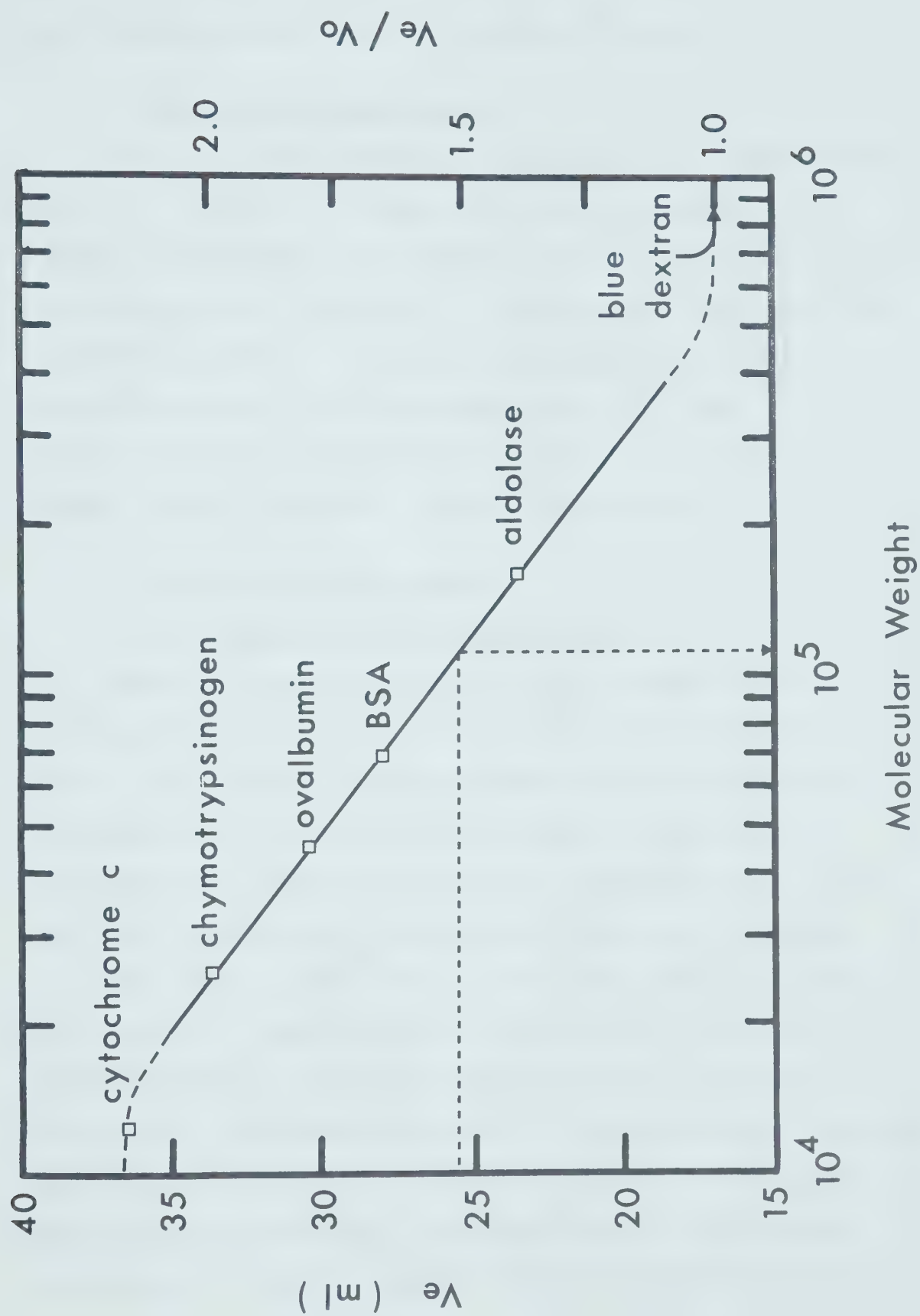


Figure 15. Estimation of the molecular weight of prothrombin by gel filtration. Column dimensions were 0.9 cm (diameter) by 60 cm (length). Samples were eluted with 0.15 M Tris-HCl buffer, pH 7.4. Flow rate was 2 ml per hour at 4° C. Sample volumes were 0.4 ml. Sample concentrations were: aldolase, 2 mg/ml; Blue Dextran 2000, 0.3%; BSA, 2 mg/ml; cytochrome c, 1 mg/ml; ovalbumin, 2 mg/ml; prothrombin, 1.24 A₂₈₀ unit. The elution position of prothrombin is indicated by the broken line.



zero concentration molecular weight of 81,000.

3. Amino acid composition

The amino acid composition determined for prothrombin is shown in Table IV. Values obtained by Seegers (11), Ingwall and Scheraga (14) and Cox and Hanahan (12) have been included for comparison. All four sets of values are in general agreement. However, the numbers of lysine, phenylalanine and serine residues found for this preparation appear high and the number for isoleucine appears low in relation to other values shown.

4. Ultracentrifuge studies

a. Sedimentation-diffusion data

Schlieren patterns obtained from an examination of prothrombin in the analytical ultracentrifuge are shown in Fig. 16. Single symmetrical peaks were observed over a concentration range of 1 to 15 mg/ml. The $S_{20,w}^0$ calculated from the average of two preparations was 5.03 S (Fig. 17). Other $S_{20,w}^0$ values which have been reported are 4.80 S (14), 4.84 S (19), 5.22 S (77) and 5.3 S (11), all obtained at pH's between 5 and 6 and in low ionic strength buffers. The data reported by Cox and Hanahan (12) at pH 7.4 are shown in Fig. 17 for comparison. Although there is good agreement between the two sets of data, the sedimentation coefficient, as determined in the present study, did not show the positive slope concentration

TABLE IV

AMINO ACID COMPOSITION OF BOVINE PROTHROMBIN

Amino Acid	Moles/ 6×10^4 g prothrombin			
	Present Study	Reference (12)	Reference (11)	Reference (14)
Lys	31.4	29.0	26.5	27.6
His	9.1	8.5	10.2	7.9
Arg	36.9	43.7	32.6	33.5
Asp	55.8	55.6	55.1	54.8
Thr	26.8	26.0	27.6	25.7
Ser	39.2	31.6	36.7	33.3
Glu	67.5	66.3	67.3	67.8
Pro	29.7	31.7	32.6	29.9
Gly	46.1	44.6	48.0	46.2
Ala	33.8	31.6	32.5	30.8
1/2 Cys	17.2	17.0	16.3	16.3
Val	30.7	32.9	34.7	31.2
Met	5.2	5.0	6.1	3.6
Ile	16.2	17.9	18.4	19.3
Leu	42.3	41.8	41.9	35.4
Tyr	16.5	18.1	19.4	14.5
Phe	21.4	18.2	19.4	17.1
Trp	11.3	11.3	11.2	14.3

Figure 16. Schlieren patterns obtained by ultracentrifugation of final prothrombin product. Sample concentration was 9.9 mg/ml in 0.001 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl and 10^{-4} M DFP. Rotor speed was 60,000 rpm; temperature 20° C. Photographs were taken at 16 minute intervals.

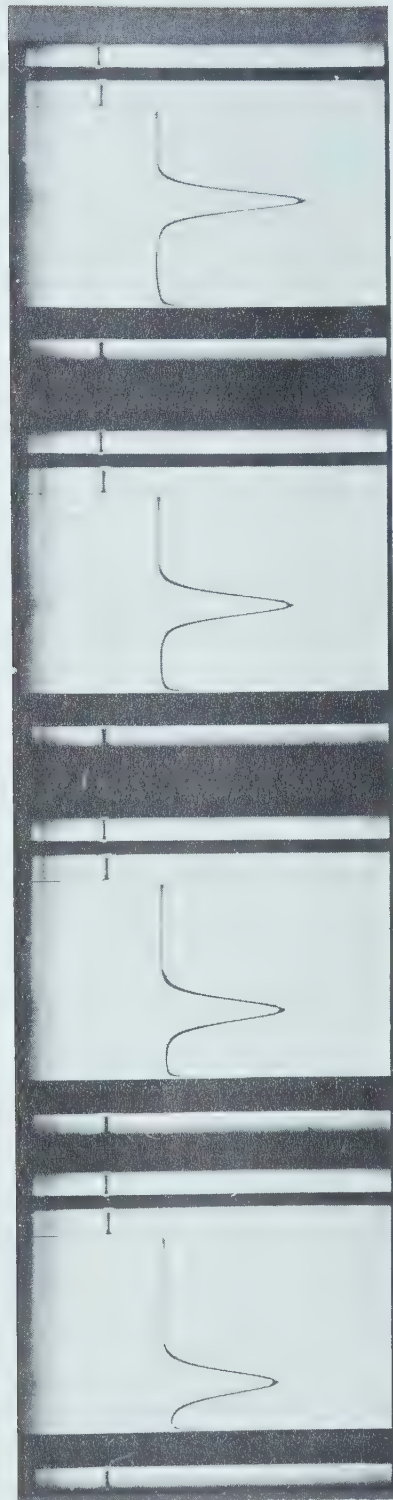
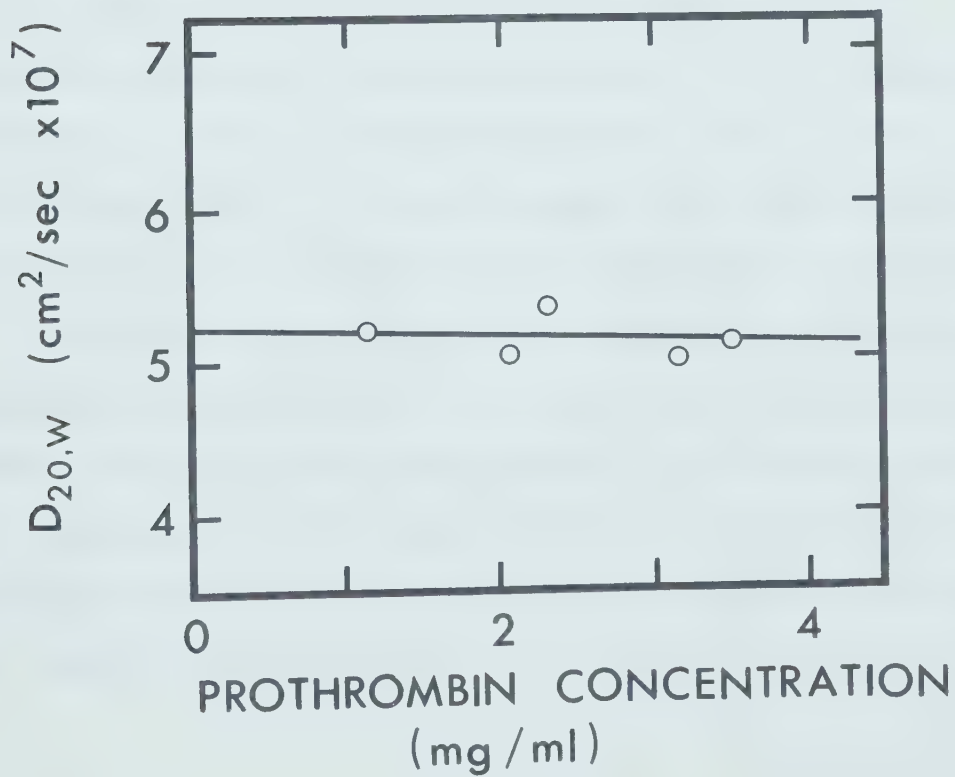
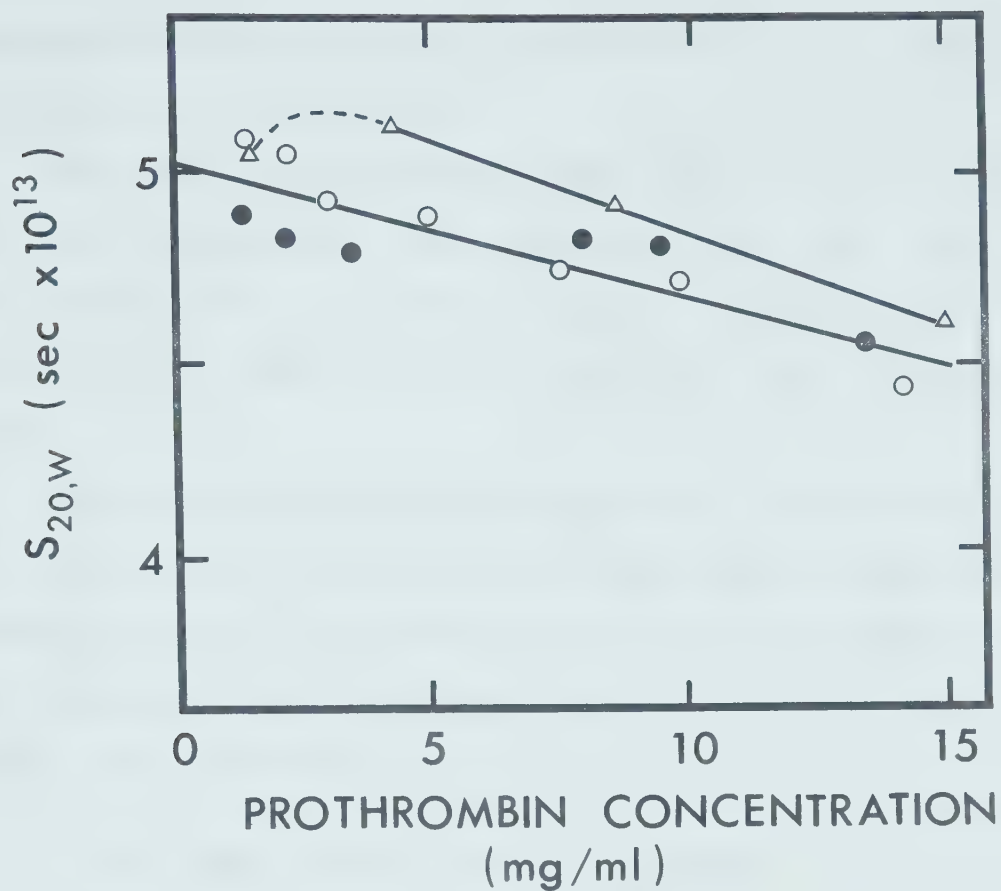


Figure 17. Concentration dependence of the sedimentation coefficient of prothrombin. Rotor speed was 60,000 rpm; temperature 20° C. Prothrombin was dissolved in 0.15 M KCl in 0.001 M Tris-HCl buffer, pH 7.4, containing 10^{-4} M DFP. ○—○ and ●—● represent different batches of prothrombin prepared by present method. △—△ represents data obtained by Cox and Hanahan (Reference 12).

Figure 18. Concentration dependence of the diffusion coefficient of prothrombin. Rotor speed was 4800 rpm; temperature 20° C. Prothrombin was dissolved in 0.15 M KCl in 0.001 M Tris-HCl buffer, pH 7.4, containing 10^{-4} M DFP.



dependence below prothrombin concentrations of 5 mg/ml as suggested by these workers.

The diffusion coefficient was found to vary little over the concentration range examined (Fig. 18). $D_{20,w}^0$ was found to be $5.22 \times 10^{-7} \text{ cm}^2/\text{sec}$. Previous values (all $\times 10^{-7} \text{ cm}^2/\text{sec}$.) are 5.6 (12), 5.27 (15), and 6.24 (19).

The molecular weight of prothrombin, calculated from the sedimentation and diffusion coefficients, using the Svedberg equation (146) was 79,000. Other values which have been reported using this calculation are 59,200 (15), 66,000 (12) and 68,000 (19).

b. Equilibrium molecular weights

Data obtained for estimation of the \bar{M}_w of prothrombin by the meniscus depletion method of equilibrium ultracentrifugation are shown in Fig. 19. \bar{M}_w values obtained in three separate experiments were 75,700, 75,900 and 75,900. Cox and Hanahan (12) have reported values of 74,200, 75,800, and 80,500 using this method.

Low speed equilibrium data analysed as a function of concentration across the cell (Fig. 20), show that the samples examined were heterogeneous. An extrapolated zero concentration \bar{M}_w value of 67,500 was obtained by combining the data for concentrations above 1 mg/ml from both sets of observations.

Figure 19. Sedimentation equilibrium determination of \bar{M}_w of prothrombin by the meniscus depletion method. Data are plotted in terms of the logarithm of fringe displacement ($y(r) - y_0$) versus radial distance squared (r^2) for prothrombin in 0.001 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl and 10^{-4} M DFP. Rotor speed was 22,000 rpm. $\square \text{---} \square$, prothrombin concentration of 0.97 mg/ml; $\Delta \text{---} \Delta$, concentration of 1.7 mg/ml; $O \text{---} O$, concentration of 3.4 mg/ml.

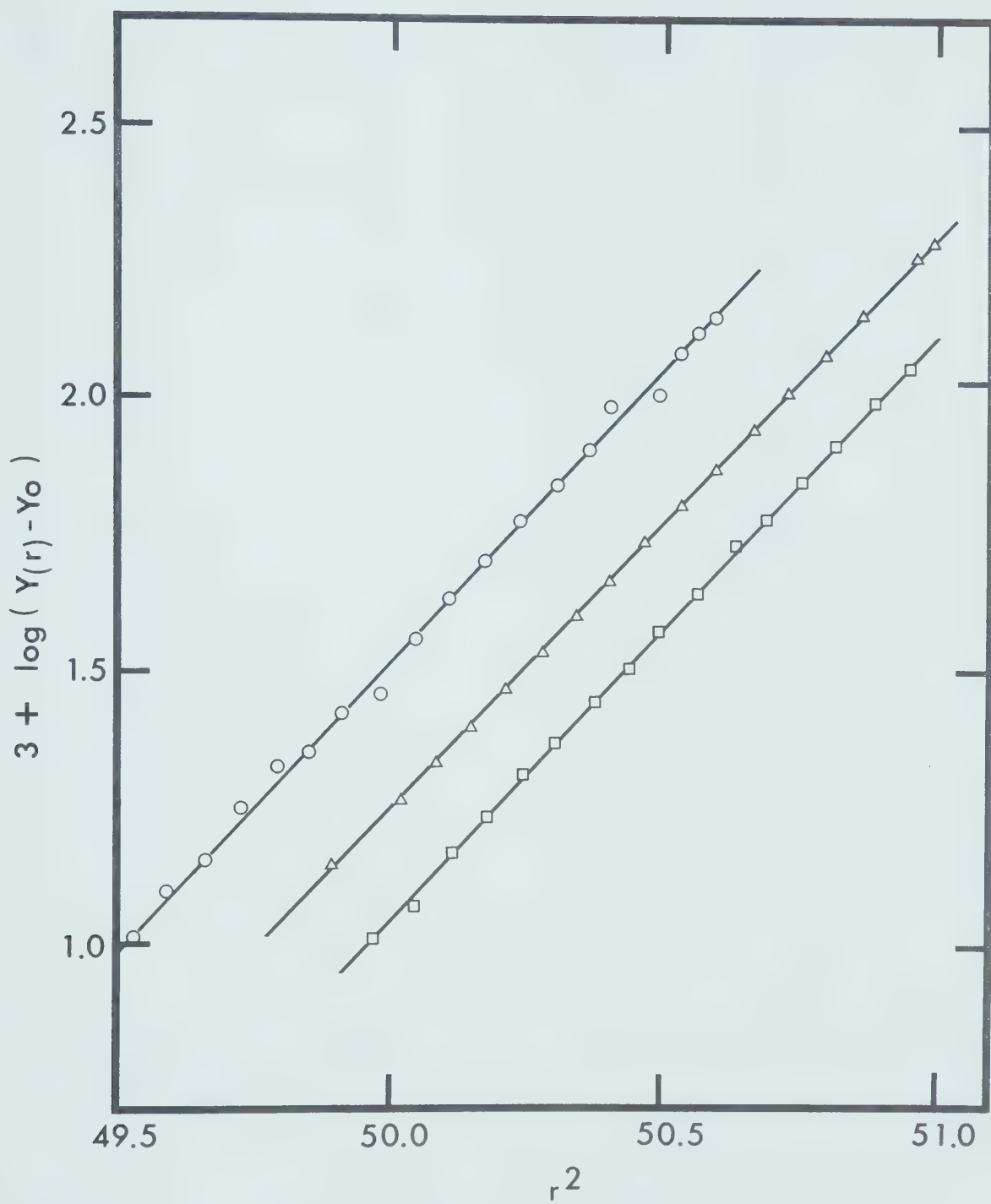

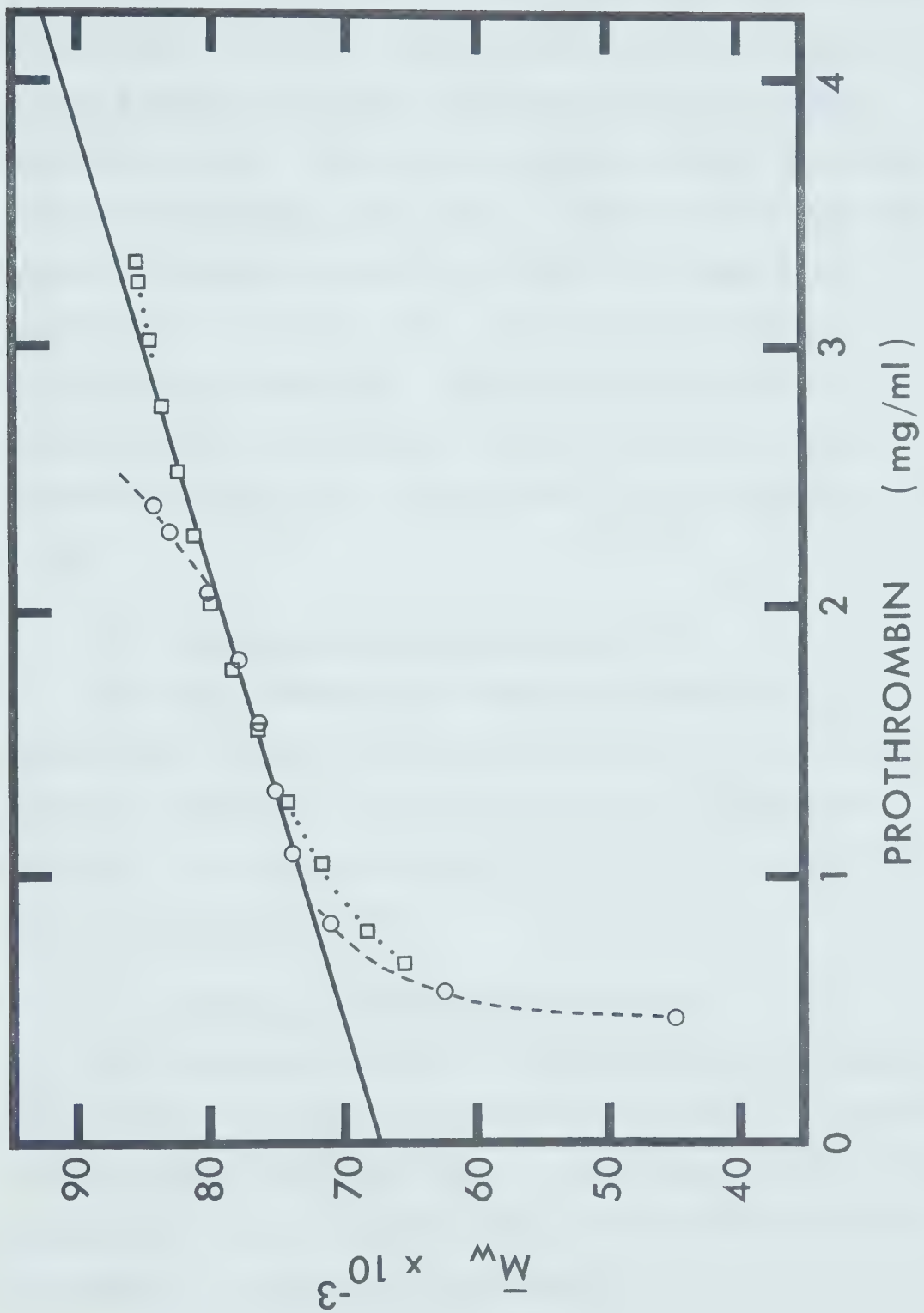


Figure 20. Low speed sedimentation equilibrium \bar{M}_w as a function of concentration. Molecular weights were calculated from the derivative of an equation fitted to $\ln y$ and concentration data. Data were analysed with the aid of an APL computer program. Prothrombin was dialysed against 0.001 M Tris-HCl buffer, pH 7.4, containing 0.15 M KCl and 10^{-4} M DFP . Rotor speed was 10,000 rpm with a 15,000 rpm initial overspeed. Equilibration time was 19 hours. Temperature was 20° C .


 O—O , prothrombin 1.08 mg/ml; □—□, prothrombin 1.54 mg/ml.



Cox and Hanahan (12) have reported similar \bar{M}_w concentration dependence in more detailed studies which suggested prothrombin dimerization at concentrations below 4 mg/ml and further polymerization at higher concentrations. The monomer molecular weight reported by Cox and Hanahan was 69,000. The value obtained from data of Ingwall and Scheraga was 68,700 when \bar{v} is adjusted to 0.70 (12, 14). The low \bar{M}_w 's observed at low concentrations (Fig. 20) may be due either to underestimation of initial sample concentrations, or possible instability of prothrombin at low concentrations (147).

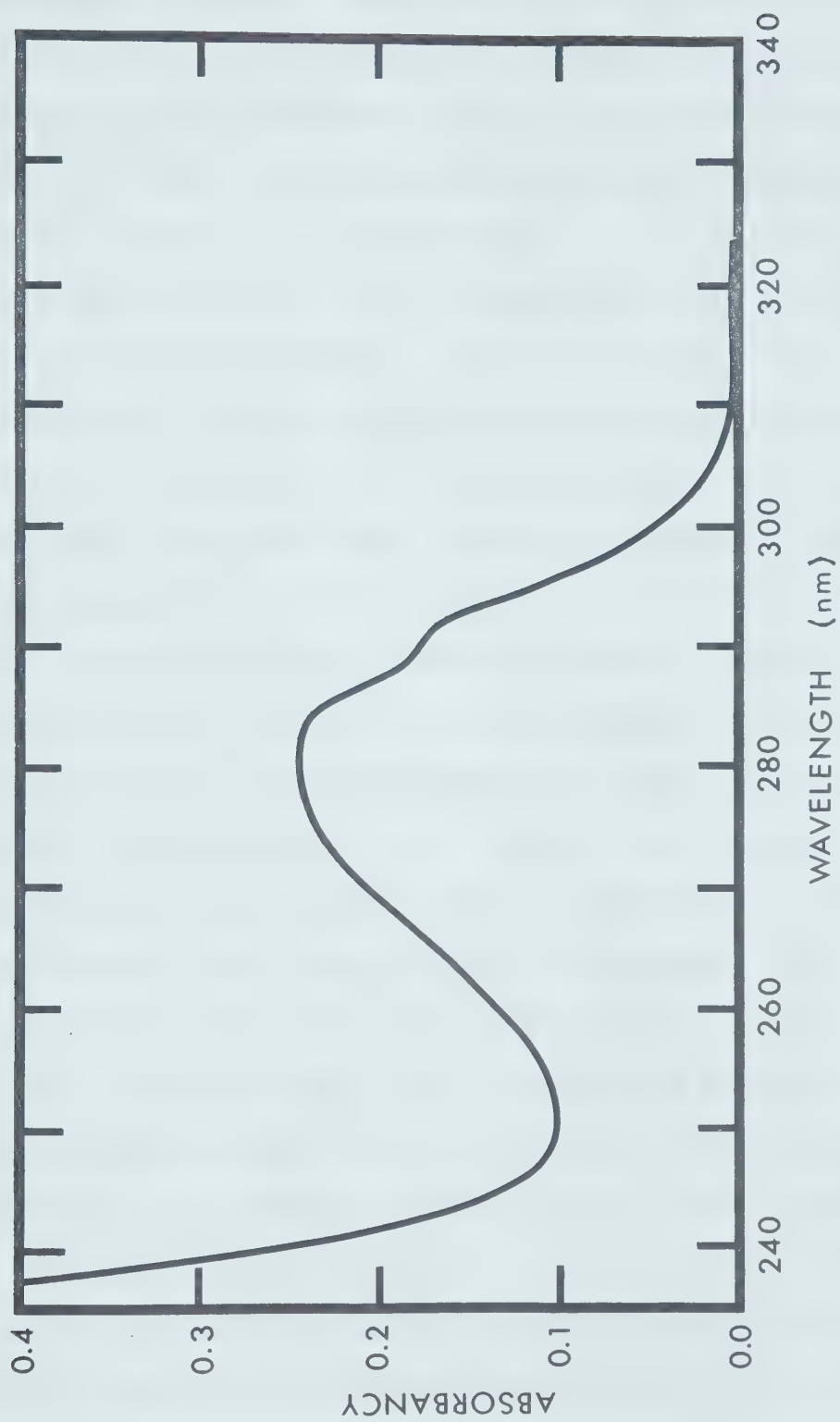
5. Ultraviolet absorption spectrum

The near ultraviolet absorption spectrum of prothrombin at pH 7.4 is shown in Fig. 21. The curve shows a broad peak around 280 nm with a shoulder at 288 nm. The secondary peak at 288 nm was not observed by Tishkoff et al. (15).

6. Specific activity and stability

The specific activity of the prothrombin prepared by this method described ranged between 1.5 and 2 standard normal plasma units per unit of absorbance at 280 nm when measured by the one-stage assay. This corresponds to a maximum of 2.8 units/mg of protein.

Figure 21. The ultraviolet absorption spectrum of prothrombin at a concentration of 0.17 mg/ml in 0.15 M Tris-HCl buffer, pH 7.4.



At present there is no universally accepted prothrombin standard. The National Institutes of Health, Bethesda, Md. have established a standard unit of thrombin activity which is defined as the amount of thrombin required to clot a standard fibrinogen solution in 15 seconds. An N. I. H. prothrombin unit is defined as the amount of prothrombin which, when activated, yields one N. I. H. unit of thrombin. There is however, no agreement on a uniform method of activating prothrombin.

Ware and Seegers (120) have defined a unit of prothrombin (the Iowa unit) which is present in oxalated bovine plasma at a level of 330 units/ml. In the present study, oxalated bovine plasma was found to contain approximately 0.5 units of prothrombin/ml. This would mean that there are approximately 660 Iowa units per unit defined in the present study. Using these figures, the specific activity for prothrombin preparations described here would be 1900 Iowa units/mg of protein. This value is well below previously reported figures of 2600 units/mg (12) and 3000 units/mg (13). Considering the diversity in assay procedures and lack of a standard reference, such comparisons are probably of relatively little value.

Factor X was not detectable in the final product. No fibrinogen clotting activity was observed in 18 hours when 14 units of prothrombin were incubated with 0.2 ml of fibrinogen (2 mg/ml) at pH 7.35 and 37° C. Samples

were not examined for possible contamination by factors VII and IX.

Prothrombin was stored at -20°C in veronal-acetate buffer, pH 6.5 containing 10^{-3} M EDTA. Samples were usually utilized within 3 months and appeared to be stable over this period.

CHAPTER IV

GEL FILTRATION OF PROTHROMBIN-PHOSPHOLIPID MIXTURES

A. Introduction

Optimal activation of prothrombin by factor Xa requires the simultaneous presence of Ca^{+2} , factor V and phospholipid (66, 85, 86). Various phospholipids or phospholipid combinations have been shown to have effects ranging from facilitation to inhibition of the prothrombin to thrombin conversion (93). The suggestion has been made that phospholipid procoagulant effects are related to the sign and magnitude of the charge on the phospholipid aggregate (97).

Previous investigations into the nature of the phospholipid requirement have shown that both factor Xa (99, 102) and prothrombin (22, 104) bind to equimolar mixtures of PS:PC in the presence of Ca^{+2} . To further examine the nature of phospholipid activity, binding studies involving prothrombin and different classes of phospholipids were carried out.

B. Methods

The binding of prothrombin to phospholipids was examined using a gel filtration technique. Chromatography of sonicated phospholipid dispersions on Sephadex G-200 columns resulted in the quantitative appearance of phospholipid in the void volume (V_0) fractions of the

eluate. Prothrombin was partially included in the gel. Binding was inferred when prothrombin activity or protein appeared in V_0 fractions.

All gel filtration studies were carried out using Pharmacia K9/30 columns (0.9 cm diameter x 30 cm height) packed with Sephadex G-200 or Sepharose 4B. Samples were eluted at 4° C with veronal-acetate buffer, normally at pH 9.0, containing either 0.010 M or 0.025 M CaCl_2 . Effluent fraction volumes were measured by weighing. Calculations of fraction weights and cumulative weights were facilitated by the use of an Olivetti-Underwood Programma 101 calculator. For the purpose of calculating elution volumes solution density was taken to be 1.00 g/ml. Fraction size ranged from 0.4 to 0.5 ml. Flow rates were 1.5 to 2.2 ml per hour.

When PC or PS:PC mixtures were to be used, the CaCl_2 concentration in the eluant buffer was 0.025 M. Column samples were prepared by mixing in order: 0.15 ml phospholipid suspension (5 mg/ml), 0.15 ml prothrombin solution (3.8 to 7.3 A_{280} units/ml) and 0.15 ml, 0.075 M CaCl_2 . When other phospholipids were used, columns were equilibrated with 0.010 M CaCl_2 in veronal-acetate buffer. In these cases, column samples were prepared by mixing in order: 0.11 ml phospholipid suspension (5 mg/ml), 0.11 ml prothrombin solution (7.3 A_{280} units/ml) and 0.22 ml 0.020 M CaCl_2 . The pH of each component solution had been

adjusted to 9.0 prior to mixing. CaCl_2 solutions were added slowly from a 500 μl syringe with constant swirling. Samples were incubated at 4° C for 20 min. before applying to the column. Applied sample size was 0.40 ml.

Fractions were analyzed directly for prothrombin activity, ^{14}C -radioactivity, and in some cases, protein. No attempt was made to disrupt any formed complexes prior to carrying out assays.

Prothrombin was assayed as outlined in the general Methods section after first adjusting the sample pH to 7.35 and the Ca^{+2} concentration to 0.025 M. The results of protein determinations were recorded as absorbance at 750 nm after correction for blank and phospholipid effects.

Radioactivity counts were converted to total phospholipid using calculated specific activities. Quenching and self-absorption effects were ignored. The phospholipid elution profiles determined by this method were the same as those observed with more concentrated samples of phospholipid measured by phosphorus determinations. The recovery of radioactivity ranged from 70% to 90%.

C. Results and Discussion

1. Preliminary experiments

Preliminary gel filtrations experiments carried out using 0.025 M CaCl_2 in veronal-acetate buffer, pH 7.35, as an eluting solution, revealed that complexing of pro-

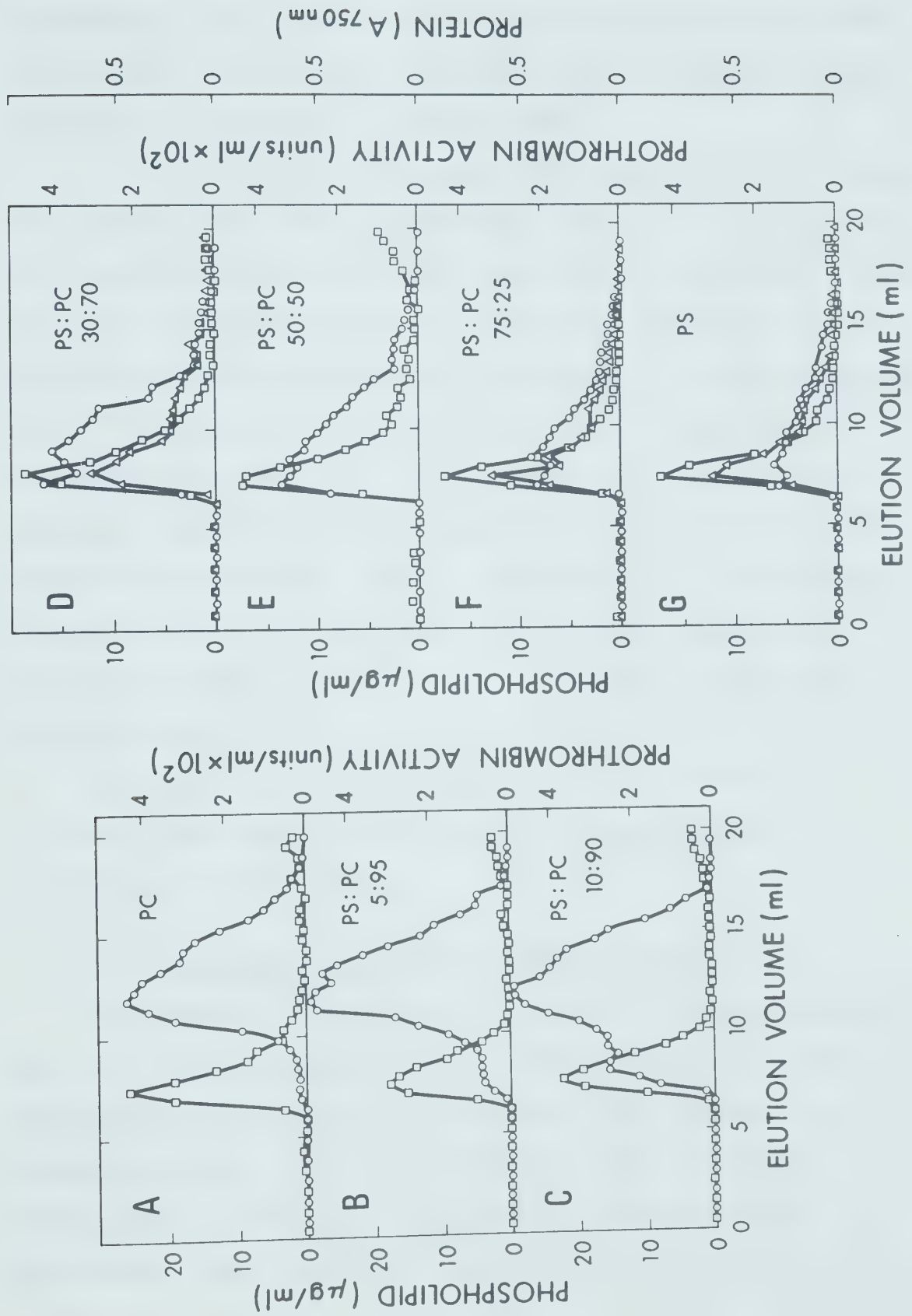
TABLE V

PRECIPITATION OF PROTHROMBIN FROM MIXTURES OF
PHOSPHOLIPID, PROTHROMBIN AND CaCl_2 AT DIFFERENT pH

Solution pH	Distribution of Total Recovered Prothrombin Activity (unit)		% of Prothrombin Activity in Precipitate
	Precipitate	Supernatant	
6.5	0.14	0.203	41
7.35	0.067	0.238	22
9.0	0.01	0.231	4

Mixtures each containing 0.5 ml of sonicated PS:PC (50/50 w/w, 5 mg/ml), 0.5 ml prothrombin solution (2.8 A_{280} units) and 0.5 ml veronal-acetate buffer containing 0.025 M CaCl_2 were set at pH 6.5, 7.35 and 9.0 and allowed to incubate at room temperature for 16 hours. Samples were then centrifuged at full speed in a clinical centrifuge (International Model CL) for 20 minutes. Precipitates were suspended in 10.0 ml veronal-acetate buffer, pH 7.35, containing 0.025 M CaCl_2 . The pH of the supernatant was adjusted to 7.35 and the prothrombin activities in the supernatants and suspended precipitates were assayed.

Figure 22. Chromatography of previously incubated mixtures of prothrombin, CaCl_2 and sonicated phospholipid dispersions on Sephadex G-200 columns. The compositions of the mixtures (total volume 0.4 ml) were as follows: A, B, and C, prothrombin 0.5 A_{280} unit, CaCl_2 0.025 M, total phospholipid 0.67 mg; D and F, prothrombin 0.97 A_{280} unit, CaCl_2 0.025 M, total phospholipid 0.67 mg; E, prothrombin 0.65 A_{280} unit, CaCl_2 0.025 M, total phospholipid 0.67 mg; G, prothrombin 0.73 A_{280} unit, CaCl_2 0.010 M, total phospholipid 0.5 mg. Phospholipid ratios are expressed as w/w. The mixtures were incubated at 4° C for 20 min. prior to column application. Columns were equilibrated, and samples were eluted with veronal-acetate buffer, pH 9.0, containing in A to F, 0.025 M CaCl_2 and in G, 0.010 M CaCl_2 . All phospholipid dispersions contained ^{14}C -PC (0.05 μg per ml) as a radioactive tracer. Final phospholipid concentrations shown were calculated from specific activities. $\text{O} \text{---} \text{O}$, prothrombin activity; $\square \text{---} \square$, phospholipid concentration; $\Delta \text{---} \Delta$, protein level expressed as absorbance at 750 nm (according to Reference 116).



protein. In the absence of any evidence for lipid-protein interaction, the recovery of activity from this column was arbitrarily set at 100%. This was done to facilitate direct comparison with further chromatograms.

It was found that increasing the proportion of PS from 0% to 30% of the total phospholipid progressively increased the amount of prothrombin activity eluted in the void volume fractions and concurrently decreased the relative proportion in the retained fractions (Fig. 22A-22D). The total recovery of prothrombin activity eluted from these columns also increased from 100% (Fig. 22A) to 184% (Fig. 22D) of that applied. Above 30% PS the amount of activity in the void volume decreased again without reappearing in the retained fractions (Figs. 22G and 22F). At the same time, total recovery of activity decreased to a minimum of 48% when PS alone was used.

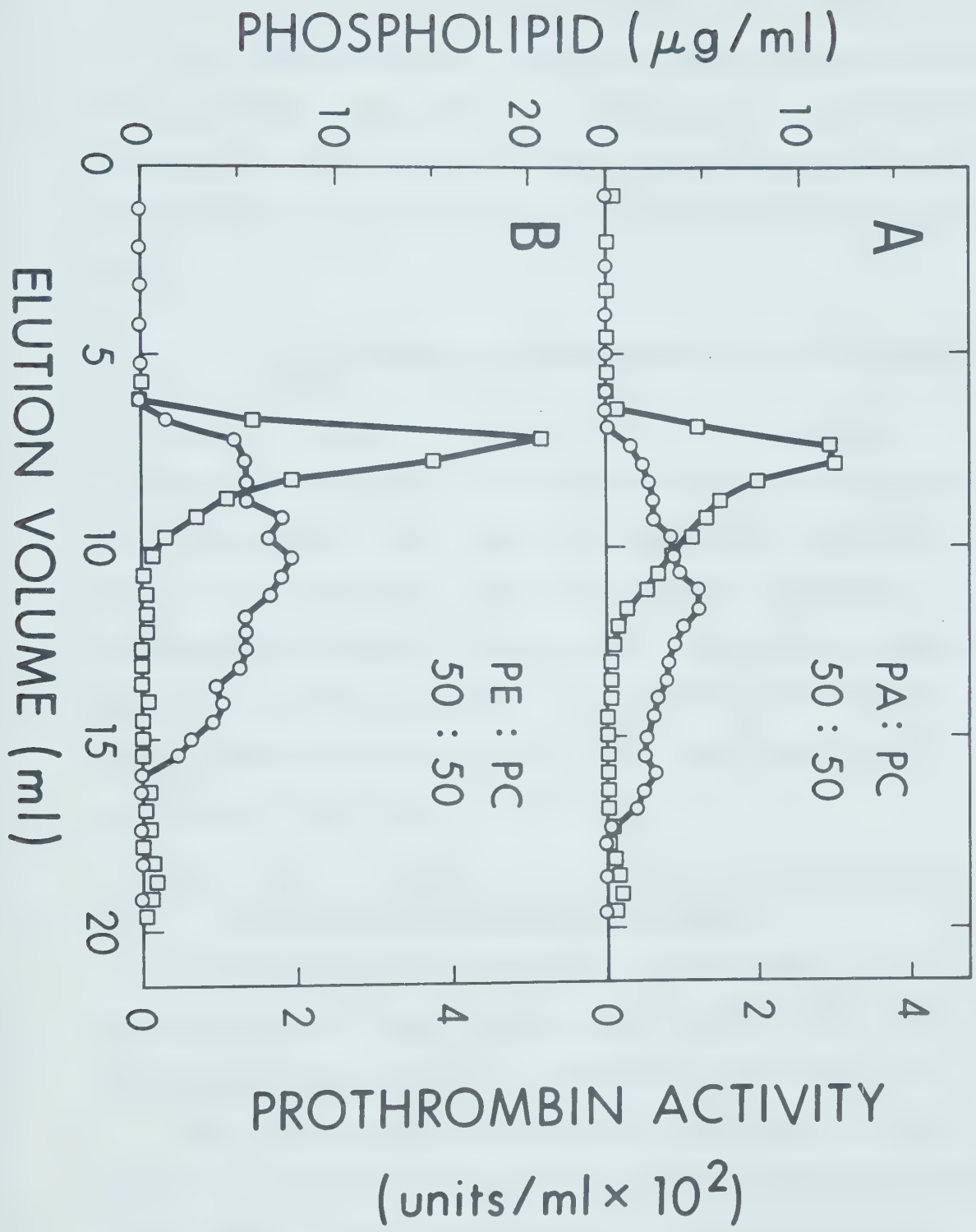
Although not shown, it was found that a 50/50 w/w PS:PC mixture bound the same amount of prothrombin at 0.010 M and 0.025 M CaCl_2 .

3. Gel filtration of prothrombin with PA:PC

In a similar manner gel filtration of a preincubated mixture containing prothrombin, 0.010 M CaCl_2 and equal weights of PA and PC was carried out. The results are presented in Fig. 23A. In contrast to the equivalent PS:PC mixture, PA:PC was ineffective in transferring prothrombin activity to the void volume fractions. Total

Figure 23. Gel filtration of prothrombin with PA:PC and PE:PC.

Chromatography of previously incubated mixtures (total volume 0.4 ml) of prothrombin (0.73 A_{280} unit), $CaCl_2$ (0.010 M), and sonicated phospholipid dispersions (0.5 ml of total phospholipid) on Sephadex G-200 columns. Phospholipid ratios are expressed as w/w. The mixtures were incubated at 4° C for 20 min. prior to column application. Columns were equilibrated, and samples were eluted with veronal-acetate buffer, pH 9.0, containing 0.010 M $CaCl_2$. All phospholipid dispersions contained ^{14}C -PC (0.05 μg per ml) as a radioactive tracer. Phospholipid concentrations were calculated from specific activities. $O \text{---} O$, prothrombin activity; $\square \text{---} \square$, phospholipid concentration; $\Delta \text{---} \Delta$, protein level expressed as absorbance at 750 nm (according to Reference 116).



activity recovered from this column was 68%.

4. Gel filtration of prothrombin with PE:PC

One lipid mixture, containing equal weights of PE and PC was tested (Fig. 23B). A rather small proportion of the applied prothrombin activity was recovered in the void volume. Total recovery of prothrombin activity was 72%.

5. Gel filtration of prothrombin with PA, PA:PS and PS

When PA alone was used only 21% of the applied activity was recovered and this was located entirely in the void volume (Fig. 24A). Progressively increasing ratios of PS:PA (Figs. 24B-24E) somewhat improved the recoveries of activity both in the void volume component and the retained component. Nevertheless the maximal total recovery (48%) was still much less than when equivalent PS:PC mixtures were used.

6. Gel filtration of prothrombin with PS:PC and PS:PA after treatment with EDTA

When preincubated samples of prothrombin, PS:PC (30:70) and Ca^{+2} were treated with 0.030 M EDTA most of the protein appeared in the retained fractions (Fig. 25A).

When preincubated samples of prothrombin, PS:PA (25:75) and 0.010 M CaCl_2 were treated with 0.030 M EDTA (Fig. 25B), a much smaller part of the total protein

Figure 24. Chromatography of preincubated mixtures (total volume 0.4 ml) of prothrombin ($0.73 A_{280}$ units), $CaCl_2$ (0.010 M) and sonicated phospholipid dispersions (0.5 ml total phospholipid) on Sephadex G-200 columns. Phospholipid ratios are expressed as w/w. The mixtures were incubated at $4^\circ C$ for 20 minutes prior to column application. Columns were equilibrated, and samples eluted with veronal-acetate buffer, pH 9.0, containing 0.010 M $CaCl_2$. All phospholipid dispersions contained ^{14}C -PC ($0.05 \mu g/ml$) as a radioactive tracer. Phospholipid concentrations were calculated from specific activities. $O \text{---} O$, prothrombin activity; $\square \text{---} \square$, phospholipid concentration; $\Delta \text{---} \Delta$, protein level expressed as absorbance at 750 nm (according to Reference 116).

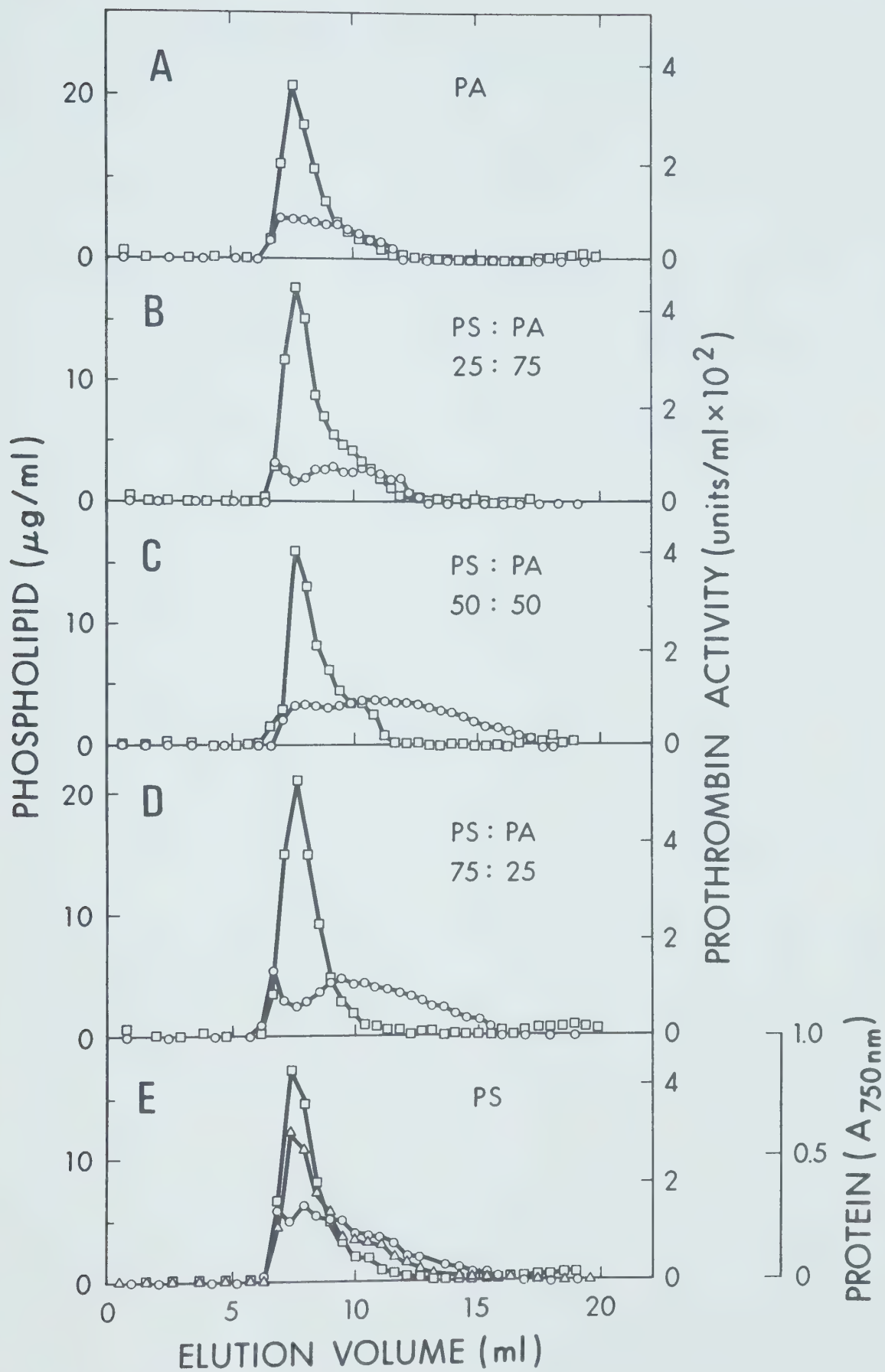
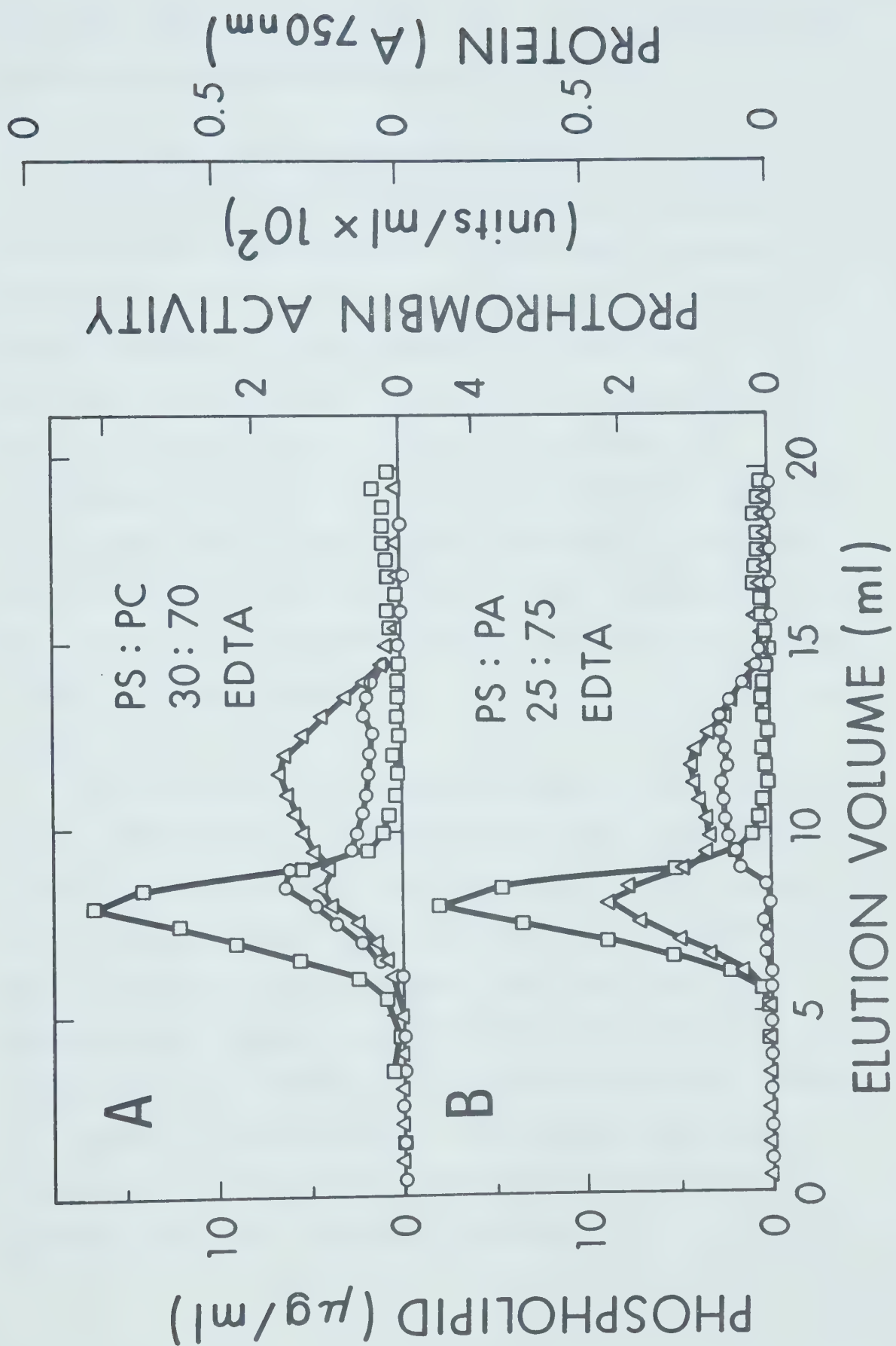


Figure 25.

Chromatography of EDTA-treated mixtures of prothrombin, CaCl_2 and sonicated phospholipid dispersions on Sephadex G-200 columns. Mixtures of 0.73 A_{280} units prothrombin (0.1 ml) CaCl_2 (0.1 ml 0.075 M) and 0.5 mg total phospholipid (0.1 ml) were incubated at 4° C for 20 minutes followed by addition of 0.1 ml 0.120 M EDTA and further incubation for 20 minutes. The mixtures were then applied to the columns. Phospholipid ratios are expressed as w/w. Columns were equilibrated and samples eluted with veronal-acetate buffer, pH 9.0. Both phospholipid dispersions contained ^{14}C -PC (0.05 $\mu\text{g/ml}$) as a radioactive tracer. Phospholipid concentrations were calculated from specific activities.

○—○ , prothrombin activity; □—□, phospholipid concentration; Δ — Δ , protein level expressed as absorbance at 750 nm (according to Reference 116).



material appeared in the free form when compared with the EDTA-treated mixtures of PS:PC.

7. Gel filtration on Sepharose 4B

It was expected that the higher molecular size exclusion limit of Sepharose 4B would afford a better resolution of the two components separated on Sephadex G-200. However, in the presence of 0.010 M CaCl_2 , it was found that mixtures containing acidic phospholipids were not eluted from the columns, even at pH 9.0. Subsequently, the gels were carefully extruded, separated into layers and the radioactivity measured in each layer. Most of the ^{14}C -label was found to have accumulated at the top of the column where the sample first contacted the gel (Table VI). This effect did not occur when PC alone was used.

It was found that mixtures of PS:PC (50/50 w/w) and PA:PC (50/50 w/w) could not be eluted from these columns in the presence of Ca^{+2} , but were eluted in the absence of Ca^{+2} . Samples containing prothrombin, PS:PC (50/50 w/w) and Ca^{+2} showed complex formation and some recovery of phospholipid. Under identical conditions, mixtures containing PA:PC (50/50 w/w) and PE:PC (50/50 w/w) showed no recovery of phospholipid. Prothrombin under these conditions was recovered in the normal total volume position with some trailing of activity.

TABLE VI
DISTRIBUTION OF ACIDIC PHOSPHOLIPID
BOUND TO A SEPHAROSE 4B COLUMN

Distance from Origin of Column (cm)	Radioactivity Measured in a 0.2 ml Aliquot of Column Material (CPM/ 0.2 ml)
0	2200
7.5	300
15	220
22.5	190
30	140

A column measuring 0.9 cm (diameter) by 30 cm (length) was packed with Sepharose 4B and equilibrated with veronal-acetate buffer, pH 8.0, containing 0.010 M CaCl_2 . A sample containing 1 mg of a 50:50 w/w mixture of PA:PC in 0.40 ml of veronal-acetate buffer, pH 8.0, and 0.010 M CaCl_2 was applied to the column. After passing 21 ml of the eluting solvent through the column, the gel was extruded from the column and 0.2 ml aliquots of Sepharose gel were removed and assayed for ^{14}C -radioactivity. The specific activity of the phospholipid mixture was 31,000 CPM/mg. The radioactivity of the eluted fractions was at background levels.

Microelectrophoretic observation of the Sepharose particles in the buffer used for chromatography revealed movement of the particles towards the anode.* This would suggest that the above results were due to a cross-linkage of acidic phospholipids to the gel via Ca^{+2} bridges.

8. Gel filtration of prothrombin in the presence of Blue Dextran 2000

During a calibration of a Sepharose 4B column, Blue Dextran 2000 and prothrombin were chromatographed together. The results of these runs are shown in Fig. 26. It is apparent that prothrombin is bound to Blue Dextran in the absence of Ca^{+2} . Blue Dextran was found to be inert in prothrombin assays.

Swart and Hemker (148) have reported an ionic strength-dependent binding of prothrombin and factors VII, IX and X to Blue Dextran. These coagulation factors were found to have different affinities for Blue Dextran. Coagulation factors could be separated by complexing them with Blue Dextran and then dissociating the complexes with increasing ionic strength buffers followed by separation using gel filtration.

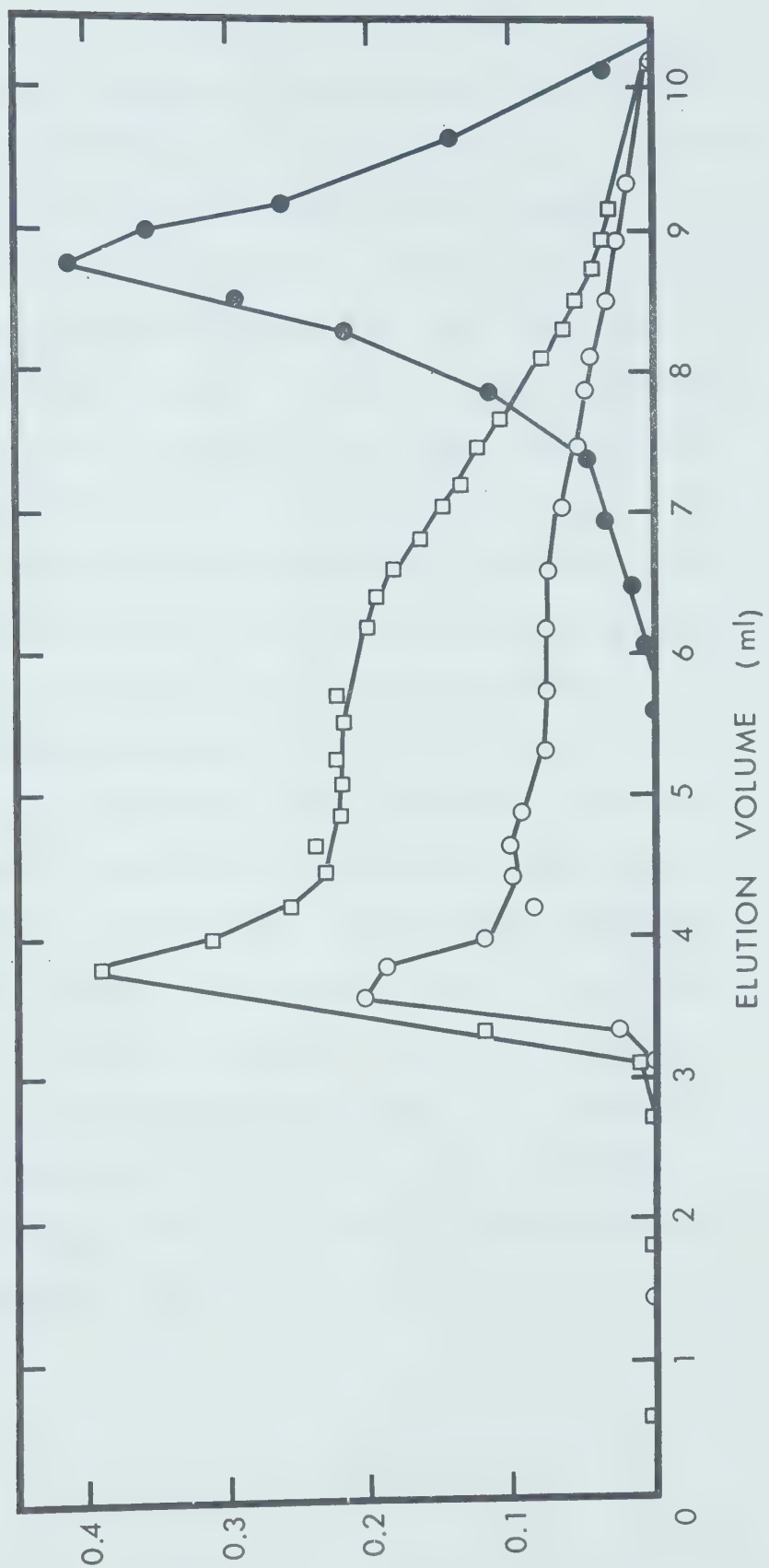
* Microelectrophoretic examination of the Sepharose particles was carried out by Dr. S. Jevons.

Figure 26. Gel filtration of prothrombin in the presence of Blue Dextran 2000. The column was packed with Sepharose 4B. Dimensions of the column were 0.9 cm (diameter) by 15 cm (length). Eluting solvent was veronal-acetate buffer, pH 7.35. Flow rate was 4 ml per hour. Sample consisted of 0.1 ml of 0.3% Blue Dextran 2000 plus 0.1 ml of prothrombin (1.2 A_{280} units).

□—□ , Blue Dextran; O—O , prothrombin activity.

The elution profile of prothrombin in the absence of Blue Dextran is shown for comparison by ●—●.

PROTHROMBIN (units/ml) & BLUE DEXTRAN ($A_{625\text{nm}}$)



D. Summary

Gel filtration studies of prothrombin-phospholipid binding in the presence of Ca^{+2} were carried out at pH 9.0. Pure PC failed to bind prothrombin in the presence of 0.025 M CaCl_2 . PS:PC phospholipid mixtures containing up to 30% PS progressively increased both the coagulant activity of the lipid and the amount of bound prothrombin. Mixtures of PS and PC containing more than 50% PS or PS alone bound approximately the same amount of protein but the complexes showed decreased activity. The amount of prothrombin bound to 50/50 w/w PS:PC mixtures was the same at CaCl_2 concentrations of 0.010 M as at 0.025 M.

PA:PC and PE:PC mixtures (50/50 w/w) were less effective than the equivalent PS:PC mixture in binding prothrombin and in recovery of prothrombin activity at a CaCl_2 concentration of 0.010 M. It was also found that a range of PA:PS mixtures, including PA and PS alone, bound prothrombin effectively. However, these complexes were much less active than those formed from PS:PC mixtures.

Attempts to disrupt PS:PA (25/75 w/w) and PS:PC (30/70 w/w) complexes with 0.010 M Ca^{+2} and prothrombin using EDTA seemed to indicate that complexes with PS:PA were more stable.

CHAPTER V

PRECIPITATION OF PROTHROMBIN - PHOSPHOLIPID MIXTURES AT pH 6.5

A. Introduction

The precipitation of prothrombin - Ca^{+2} - phospholipid complexes at lower pH levels, as reported in the previous chapter, provided an alternative but complementary method of evaluating binding.

It was found that mixing acidic phospholipids with 0.01 M CaCl_2 at pH 6.5 resulted in the formation of precipitates which could be readily separated by centrifugation. Under specified conditions, some prothrombin could be recovered with ^{14}C - labelled phospholipids in such precipitates. These observations formed a basis for the analysis of insoluble complexes of prothrombin with different phospholipid mixtures.

B. Methods

Solutions were prepared by mixing, in order, 0.20 ml each of sonicated phospholipid (containing a ^{14}C - PC tracer), pH 6.5 (5 mg/ml), prothrombin solution, pH 6.5 (7.3 A_{280} units/ml), and 0.030 M CaCl_2 in veronal-acetate buffer, pH 6.5. The CaCl_2 solution was added carefully through a syringe with constant mixing. Control solutions were prepared in the same manner except that the prothrombin solution was replaced by an equivalent volume of veronal-acetate buffer, pH 6.5. Compositions of the phospholipid

mixtures are reported in terms of percentage weights.

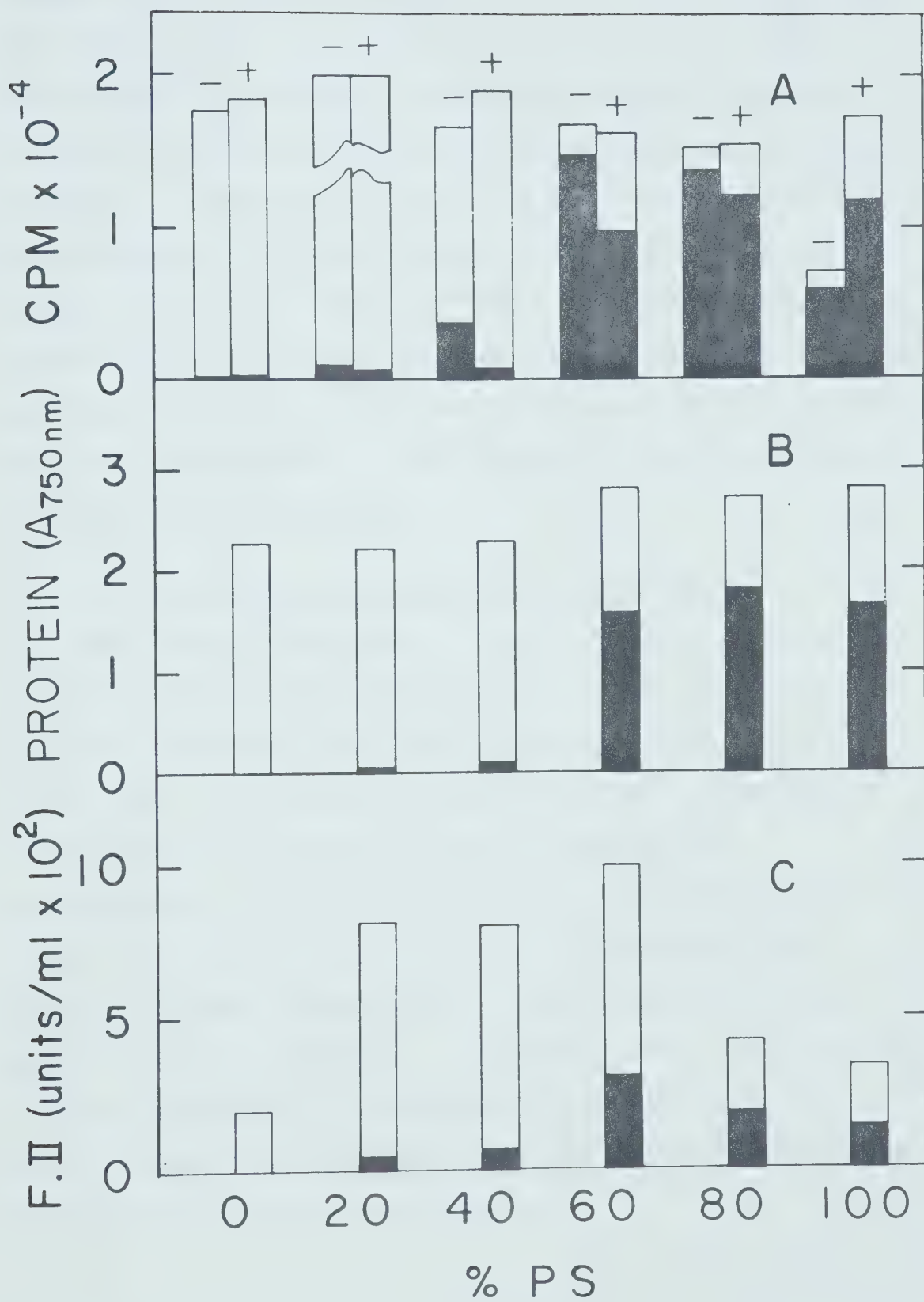
Samples were allowed to remain at 4° C for approximately 24 hours and were then centrifuged at full speed in a clinical centrifuge (International Model CL). The supernatants were removed and the precipitates washed twice with 0.2 ml of 0.010 M CaCl₂ in veronal-acetate buffer, pH 6.5. Precipitates were then suspended in 1.0 or 2.0 ml (depending on the amount of precipitate) of 0.010 M CaCl₂ in veronal-acetate buffer, pH 9.0. Supernatants and precipitate suspensions were then analysed for protein (116), prothrombin activity and ¹⁴C-radioactivity.

C. Results and Discussion

1. Precipitation of prothrombin and PS:PC

Fig. 27 shows the distribution of ¹⁴C-radioactivity, protein material and prothrombin activity between the supernatant and washed precipitates when PC, mixtures of PS with PC and PS alone were used. It can be seen (Fig. 27A) that negligible amounts of lipid precipitated when the content of PS in the mixtures was equal to or less than 20% of the total phospholipid. At 40% PS significant precipitation was observed in the absence of added prothrombin but when the protein was present this seemed to afford some protection against precipitation. At 60% PS or higher most of the lipid was precipitated under these conditions in the presence or absence of prothrombin. Fig. 27B shows that about 60% of the total protein present

Figure 27. Distribution of (A) ^{14}C -radioactivity, (B) protein (determined according to Reference 116), and prothrombin activity (C) in the precipitates and supernatants from mixtures of sonicated PS:PC dispersions (1.0 mg total phospholipid in each mixture with prothrombin (1.46 A_{280} units) and CaCl_2 (0.010 M) at pH 6.5. Compositions of the phospholipid dispersions are expressed as w/w, and all mixtures contained ^{14}C -PC (0.05 μg) as a radio-active tracer. The preparation of these mixtures is described in the text. Closed portions of the histograms represent precipitates while open sections denote supernatants. Mixtures prepared in the presence of prothrombin are indicated by (+), while those prepared in its absence are designated (-).

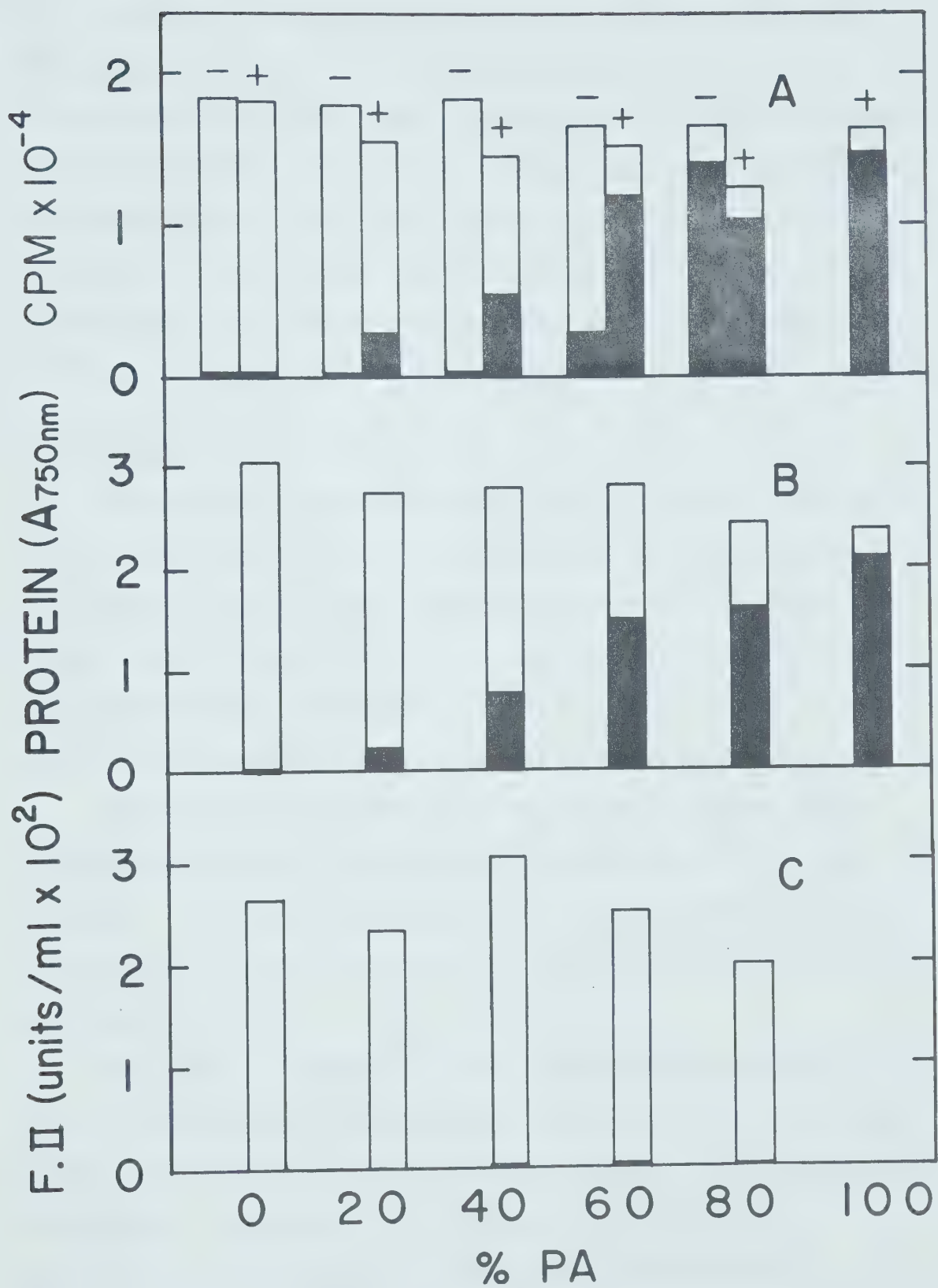


was also precipitated along with the lipids. Fig. 27C shows that the total coagulant activity measurable in the supernatant plus precipitate increased with increasing percentage PS up to 60%, then decreased again. This was also true of activity present in the precipitate alone. Therefore, comparison of Figs. 27B and 27C shows that protein bound with phospholipid in the precipitate is unable to manifest optimal activity when the percentage PS exceeds 60% of the total phospholipid present. It is also apparent from Fig. 27 that the relative amounts of protein and prothrombin activity were the same in both the precipitates and the supernatants.

2. Precipitation of prothrombin with PA:PC

The results are shown in Fig. 28. Again there was negligible phospholipid precipitation when the acidic species constituted less than 20% of the total lipid (Fig. 28A). At levels of 40% PA and 60% PA the presence of prothrombin appeared to enhance phospholipid precipitation. The protein material itself was found largely in the precipitates at 60% PA and higher and appeared to bind slightly more effectively than to the equivalent PS:PC mixtures. In spite of this, the coagulant activity localized in these precipitates was very low. When PA alone was used no activity was found either in the precipitate or in the supernatants.

Figure 28. Distribution of (A) ^{14}C -radioactivity, (B) protein (determined according to Reference 116), and prothrombin activity (C) in the precipitates and supernatants from mixtures of sonicated PA:PC dispersions (1.0 mg total phospholipid in each mixture with prothrombin (1.46 A_{280} units) and CaCl_2 (0.010 M) at pH 6.5. Compositions of the phospholipid dispersions are expressed as w/w, and all mixtures contained ^{14}C -PC (0.05 μg) as a radio-active tracer. The preparation of these mixtures is described in the text. Closed portions of the histograms represent precipitates while open sections denote supernatants. Mixtures prepared in the presence of prothrombin are indicated by (+), while those prepared in its absence are designated (-).



3. Precipitation of prothrombin and PE:PC

A single, 50/50 w/w mixture of PE:PC was examined. The distribution of recovered protein was 5% in the precipitate and 95% in the supernatant. The distribution of phospholipid was also 5% in the precipitate and 95% in the supernatant. The total recovery of prothrombin activity was 0.037 unit, 10% of which was in the precipitate. In the control sample 2% of the recovered ^{14}C -radioactivity was in the precipitate.

D. Summary

Mixtures of prothrombin and various combinations of PA:PC, PE:PC and PS:PC in the presence of CaCl_2 , at pH 6.5, were found to form precipitates which contained phospholipid and prothrombin. The amounts of protein precipitated were dependent largely on the extent to which the phospholipids themselves were precipitated.

The levels of prothrombin activity recovered were maximal with PS:PC mixtures containing between 20% and 60% PS. For any given phospholipid mixture the specific activity was almost identical in the precipitate and in the supernatant.

The total activity of prothrombin recovered from PA:PC mixtures was, in all cases, less than that recovered from PS:PC mixtures. PA alone was found to be completely inhibitory. Although the amounts of protein which appeared in the precipitates of PA:PC mixtures were

greater than those amounts for the corresponding PS:PC mixtures, precipitates containing PA had almost no prothrombin activity.

A single 50/50 w/w PE:PC mixture which was examined was found to show the same relative distribution of protein and prothrombin activity between supernatant and precipitate as observed with PS:PC mixtures. The recovery of prothrombin activity was greater than that found in mixtures of PA with PC and corresponded closely to the recovery observed with an 80% mixture of PS in PC or PS alone.

CHAPTER VI

STUDIES ON THE EFFECT OF Ca^{+2} ON THE ULTRAVIOLET ABSORPTION SPECTRUM AND SEDIMENTATION RATE OF PROTHROMBIN

A. Introduction

Ca^{+2} is essential both in the binding of prothrombin to phospholipids and in the conversion of prothrombin to thrombin. Ganrot and Nilehn (149) observed a reduction in the electrophoretic mobility of prothrombin in the presence of 0.0025 M calcium lactate and suggested that Ca^{+2} is bound to prothrombin. Hanahan et al. (22) have reported the lack of an ultraviolet difference spectrum for prothrombin in the presence of 0.001 M CaCl_2 , and have briefly mentioned a difference in light scattering properties.

Experiments of a preliminary nature were carried out to ascertain whether 0.010 M CaCl_2 had any effect on the sedimentation coefficient of prothrombin or resulted in the appearance of an ultraviolet difference spectrum.

B. Methods

Samples of prothrombin (2 ml) were dialyzed at 4° C for 24 hours against 3 changes of 5 litres of the appropriate buffer containing 10^{-4} M DFP.

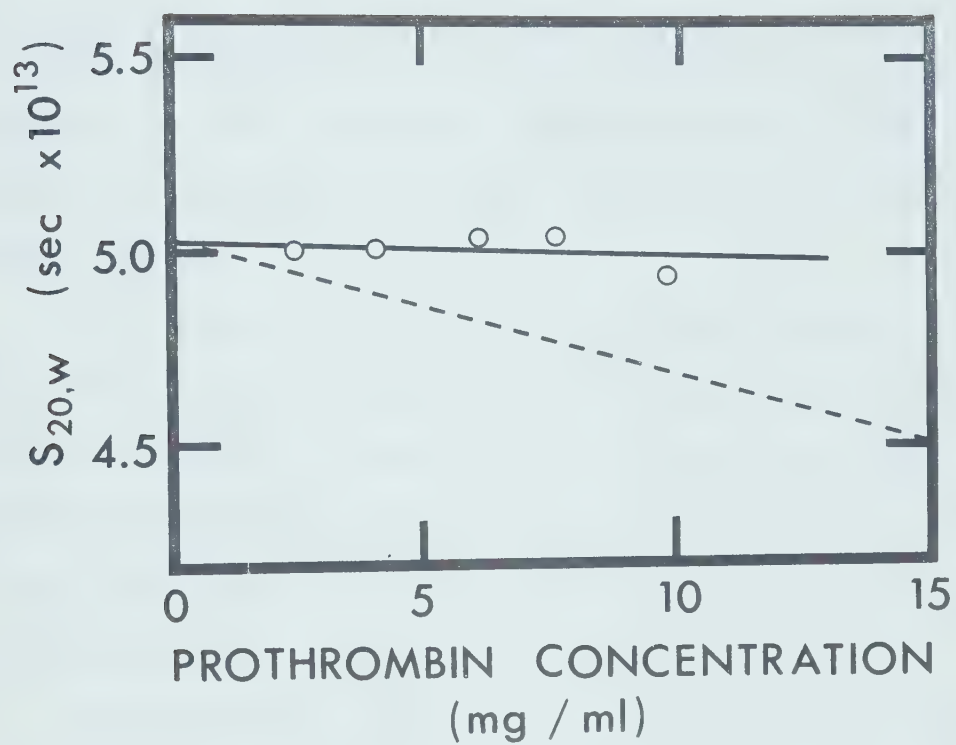
For the sedimentation velocity studies, prothrombin solutions were dialyzed against 0.001 M Tris-HCL buffer, pH 7.4, containing 0.12 M KCl and 0.010 M CaCl_2 . For control experiments the same buffer containing 0.15 M KCl but no CaCl_2 was used. Ultracentrifugation was carried out at 20°C and 60,000 RPM. Schlieren patterns were photographed at 16 minute intervals except in the cases of sample concentrations below 2 mg/ml when photographs were taken at 8 minute intervals. Measurements were made on 6 to 8 pictures.

Difference absorption spectra were obtained at 25°C using solutions of prothrombin in 0.15 M Tris-HCL buffer, pH 7.4. The method used was similar to that of Sipos and Merkel (150). Prothrombin solution (0.95 ml containing 1.2 to 2.5 mg/ml) was placed in both sample and reference cells. A baseline was obtained by scanning the wavelength range from 340 to 240 nm. 10 μl of 3 M NaCl in 0.15 M Tris-HCL buffer, pH 7.4, was then added to the reference cell and 10 μl of 1 M CaCl_2 , in the same buffer added to the sample cell. Solutions were mixed gently with Teflon mixing sticks and the difference spectrum was measured.

C. Results and Discussion

The effect of 0.010 M CaCl_2 on the sedimentation coefficient is shown in Fig. 29. The relationship between $S_{20,w}$ and concentration (c) in mg/ml for the

Figure 29. The effect of CaCl_2 on the sedimentation coefficient of prothrombin. Prothrombin solutions were dialyzed against 0.001 M Tris-HCl buffer, pH 7.4, containing 0.12 M KCl and 0.010 M CaCl_2 . Ultracentrifugation was carried out at 60,000 rpm and 4° C. The broken line represents the data obtained in the absence of CaCl_2 (from Figure 17).



CaCl₂-containing sample is:

$$S_{20,w} = 5.02 - 0.0024 c$$

and

$$S_{20,w} = 5.03 - 0.034 c$$

for the KCl reference. The decreased slope in the presence of CaCl₂ would be consistent with a Ca⁺²-induced conformational change resulting in a decreased axial ratio.

The presence of CaCl₂ in prothrombin solutions resulted in the appearance of small positive difference spectra relative to NaCl controls (Table VII). The differential absorption was observed in the 287-293 nm range which may be assigned to tyrosine and tryptophan perturbations.

Similar small, positive difference spectra have been observed in studies with trypsin in the presence of CaCl₂ (150). The results in the case of the trypsin study were supported by studies of optical rotatory dispersion power, sedimentation rates, gel filtration characteristics and electrophoretic mobility of trypsin in the presence and absence of Ca⁺² and were interpreted in terms of the formation of a calcium-trypsin complex.

TABLE VII

ULTRAVIOLET DIFFERENTIAL ABSORPTION OF
PROTHROMBIN IN THE PRESENCE OF CaCl_2

Concentration of Prothrombin (mg/ml)	Differential Absorption in the 287-293 Region (absorbance units)
1.15	0.012
1.6	0.015
2.5	0.022

Difference spectra were obtained by placing 0.95 ml of prothrombin solution in both sample and reference cells. A baseline was obtained by scanning the wavelength range of 340 to 240 nm. 10 μl of 3 M NaCl in 0.15 M Tris-HCl buffer, pH 7.4, was then added to the reference cell and 10 μl of 1 M CaCl_2 in the same buffer added to the sample cell. Positive difference spectra were observed in the region of 287 to 293 nm.

The altered form of trypsin which was produced possessed greater enzymic activity than calcium-free trypsin and was more heat-stable. In view of the previously reported effect of Ca^{+2} on the electrophoretic mobility of prothrombin (149), and the observed effect of CaCl_2 on the sedimentation coefficient (Fig. 29), some type of complexing with Ca^{+2} is suggested in the case of prothrombin. Recent investigations in this laboratory using an equilibrium dialysis technique indicate that prothrombin does bind significantly more Ca^{+2} than a bovine serum albumin control sample.

In summary, it appears that prothrombin is able to bind Ca^{+2} ions as reflected by changes in sedimentation behaviour and ultraviolet absorption. As a result, the molecule probably becomes more compact and this could be due to intramolecular Ca^{+2} bridges. Some of the same binding sites may be involved in intermolecular Ca^{+2} bridges during formation of prothrombin- Ca^{+2} -phospholipid complexes.

CHAPTER VII

DISCUSSION

The purification of prothrombin from bovine blood has presented formidable problems for nearly half a century. While many refinements in the procedures employed have been introduced, neither the physico-chemical nor the biological criteria for purity have been satisfactorily met. Although the products reported by several investigators appeared homogeneous when examined by sedimentation-velocity centrifugation (12, 117, 135, 137), gel filtration (12, 15, 117) and electrophoretic methods (12, 14, 117, 137), contamination at the level of 1-2% could not be discounted. Since several other clotting factors, including factors VII, IX and X, have physical and chemical properties closely resembling those of prothrombin and since trace amounts of these factors profoundly affect the biological activity of prothrombin preparations, the slightest degree of heterogeneity assumes considerable importance.

Several aspects of the reported properties do not appear entirely satisfactory when examined critically in terms of protein homogeneity of the preparations. While disc electrophoresis at pH 8.6 demonstrated the presence of a single major component, the stained band always appeared somewhat diffuse regardless of the loading

factor. It appeared therefore that there was some alteration in the physical or chemical properties of this material during electrophoresis. Again, molecular weight estimates for prothrombin have varied quite considerably depending on the method used. Thus, sedimentation velocity coupled with rate of diffusion measurements have uniformly yielded values of \bar{M}_w close to 68,000 (12, 19, 152). In contrast, high speed sedimentation-equilibrium experiments reported in Chapter III gave \bar{M}_w values of 75,000 while low-speed equilibrium runs gave non-linear plots of $\ln c$ versus r^2 in which low concentration regions of the cell showed \bar{M}_w 's from 55,000 to 65,000 whereas high concentration extremes indicated \bar{M}_w 's of 70,000 to 85,000. Similar results have been obtained with preparations of prothrombin reported by other workers (12, 14, 15). Tishkoff et al. (15) however, did obtain linear plots with prothrombin in the presence of 6 M guanidine hydrochloride and 0.5% mercaptoethanol and obtained a best fit \bar{M}_w of 70,477. Nonetheless, they subsequently demonstrated that their material was in fact a family of glycoproteins comprising prothrombin and factors VII and X. A molecular weight of $84,000 \pm 3,000$ was obtained by SDS-acrylamide electrophoresis and about 110,000 by gel filtration on Sephadex G-200

calibrated with ovalbumin, cytochrome c, aldolase, chymotrypsinogen, Blue Dextran and sodium dichromate. The last figure may be corrected to about 85,000 by comparison with other glycoproteins measured in this way since these substances tend to be retained more strongly than other proteins by the gel (129).

A further problem concerns the carboxyl- and amino-terminal residues of prothrombin. A procedure for prothrombin purification developed by Seegers (135), which did not involve a chromatographic step, yielded material with N-terminal alanine and C-terminals tyrosine and glycine. However, with prothrombin chromatographed on DEAE-cellulose only one C-terminal residue was found and this was serine (16, 17). It was claimed that DEAE-cellulose chromatography altered the structure of the prothrombin molecule as well as its biological activity. It is difficult to see how DEAE-cellulose itself could directly cause proteolytic cleavage of a polypeptide chain but it is possible that adsorption to the ion-exchange groups could promote proteolysis by a contaminant enzyme also adsorbed at the same site. Thus, the findings with respect to C-terminal changes might best be interpreted by assuming that the starting material was heterogeneous.

Next it will be useful to consider the various assay procedures used to quantitate the biological activities of these preparations. These all involve conversion of the prothrombin to thrombin which is then available for initiation of clot formation, utilizing fibrinogen as substrate. All assays are designed to render the conversion of prothrombin to thrombin rate-limiting. This can be achieved either by use of a prothrombin-deficient substrate plasma saturated with respect to other clotting factors (one-stage assay) or alternatively by first converting all the available prothrombin to thrombin in the absence of fibrinogen and then separately measuring the rate of clot formation when added to fibrinogen (two-stage assay). In practice, both methods suffer disadvantages due to inability to meet the theoretical specifications for levels of the different clotting factors. Thus, the measured activities tend to reflect the extent of contamination by those factors involved in prothrombin activation. In our experience, the method least susceptible to this error is the modified one-stage assay of Hjörst et al. (118) in which Russell's Viper venom is used as activator and the only accessory clotting factors subsequently involved are factors V and X. However, while it is certainly desirable to use more

than one assay system to evaluate the specific activity of the final product, this has not usually been done. The use of different units by different investigators also precludes exact comparisons of different prothrombin preparations.

Thus, with regard to the purity of the prothrombin preparations used in the experiments reported herein, although they appear close to homogeneity, some uncertainty still exists as to the exact extent of contamination with other clotting factors. They appear comparable to the preparations of several other investigators in this respect. With this in mind, an attempt was made to design experiments in which bulk physicochemical changes were observed which would be nearly independent of minor contamination of the prothrombin preparations.

Gel filtration at pH 9.0 followed by analysis of one-stage prothrombin activity, protein, and ^{14}C radioactivity gave results which reflected both the extent of binding of prothrombin to phospholipids as well as the level of activity in the lipid-protein complexes formed. Precipitation at pH 6.5 was used for making additional comparisons of protein and one-stage prothrombin activity in the complexes. The disadvantage suffered in the latter technique was that the amounts of protein precipitated were dependent on the extent to which the phospholipids themselves were precipitated.

When the data obtained from both methods were compared, however, valid conclusions could be drawn with regard to the extent of binding and the level of the activity of each of the complexes.

It has been known for some years that certain combinations of phospholipids can act as substitutes for platelets during blood coagulation, whereas other combinations are ineffective (93). It seems probable that the acceleratory effect of phospholipids on blood coagulation is dependent on their ability to provide a catalytic surface in the aqueous medium for protein-protein interactions. Conflicting data regarding the requirement for a specific phosphatide species or specific combinations of phosphatides appeared to be uniquely resolved by the correlation of procoagulant activity with the sign and magnitude of the surface charge on the lipid particles (95, 97, 98). This hypothesis was based mainly on the demonstration of a relationship between the activity of various phosphatides in clotting tests and their negative electrophoretic mobility. Some deviations from this generalization were subsequently reported, however (96, 99). In particular, PA previously mixed with PC was quite ineffective compared with PS:PC at any given electrophoretic mobility. Furthermore, several authors have reported that phosphatidyl serines exhibit

procoagulant activities at low concentrations but anticoagulant activities at higher concentrations, especially when solubilized with sodium deoxycholate or serum albumin (153-157). No satisfactory explanation for this finding has been given hitherto.

Pure PC is essentially inert in clotting systems (93) and failed to bind prothrombin in the presence of 0.025 M Ca^{+2} ions (Fig. 22A). A mixture containing equal amounts of PE and PC showed only a marginal increase in the extent of binding of prothrombin (Fig. 23B). Progressively increasing the percentage of PS mixed with PC up to 30% PS increased both the coagulant activity of the lipid and the amount of bound prothrombin (Fig. 22, B to E). When PS:PC mixtures containing more than 50% PS were used or when PS alone was used, approximately the same amount of prothrombin was bound to the lipid as before but the complexes showed decreased activity (Figs. 22, F and G, 27B and 28B). To account for these results it is suggested that a loose binding of prothrombin to lipids is necessary for conversion of prothrombin to thrombin, but that when the protein is more firmly attached either this conversion cannot take place or the thrombin formed cannot be released from the lipid surface and thus remains inactive.

Since PA cannot substitute as a procoagulant for PS in mixtures with PC, binding of prothrombin to PA and to PA:PC mixtures, was next studied. It was found that these lipids also bound protein quite effectively (Figs. 25A and 28B), but the complexes were even less active (Figs. 24A, 25A, and 28C) than when PS alone was used. Thus, PA may have even more affinity for prothrombin than has PS at a given charge density. Several explanations may be offered as to why this should be so. (a) The extent of binding may be determined by the presence of specific chemical groups such as phosphate, carboxyl, or amino groups; (b) the size and shape of the lipid aggregates or their molecular architecture may be affected by the phospholipid composition; (c) the approach of a protein macromolecule to the lipid surface may be restricted by the bulkier phosphoserine headgroups of PS in the interface as compared with the more compact phosphate groups of PA.

Since PA and PS both carry one to two net negative charges per molecule at pH 7.35, it was not expected that varying the proportions of these two species in binary mixtures would greatly affect their electrophoretic mobility. This was found to be the case (158). The ability of such mixtures to substitute for Cephalin in the one-stage test (118) was found to be independent

of the electrophoretic mobility and was related only to the amount of PS present in each mixture. In contrast with PC which potentiated the coagulant activity of PS, PA exerted a small but definite inhibitory effect on PS. This again could be related to the greater ability of PA to bind free prothrombin in the form of an inactive complex. Careful inspection of Fig. 24, A to E, shows that increasing the percentage of PA in PA:PS mixtures removes progressively more of the free prothrombin component into a low activity complex.

The tighter binding of prothrombin to PS:PA as compared with PS:PC was confirmed by treating the complexes with EDTA. Although in both cases this treatment caused an apparent loss of activity of prothrombin, probably due to chelation of Ca^{+2} in the subsequent clotting assay, a significant difference in the amounts of protein transferred back to the free form was evident. Whereas the complex containing PS:PC (30:70) retained only about 20% of the total protein present (Fig. 25A), the complex containing PS:PA (25:75) retained over 50% after EDTA treatment (Fig. 25B). This may indicate that, in complexes involving PA, either Ca^{+2} incorporated into the lipid-protein complex is more firmly bound or bonds not involving Ca^{+2} are formed.

The experiments with Sepharose 4B also shed some light on the type of binding involved in formation of these complexes. The gel is prepared from agar and may contain residual agarose with carboxyl and sulphate groups (159-161). This suggestion is supported by the negative electrophoretic mobility of the beads. Presumably, acidic phospholipids can then cross-link to the gel via Ca^{+2} bridges. The failure of the gel to bind the lipid-protein complexes suggests that a similar type of mixed chelate may be involved in stabilizing these complexes. PC itself binds Ca^{+2} , only weakly (162) and also did not attach to Sepharose 4B or bind prothrombin.

This interpretation receives some support from the data in Chapter VII in which changes in the sedimentation coefficient and ultraviolet absorption spectrum of prothrombin were observed in the presence of CaCl_2 . These suggest that prothrombin can interact with Ca^{+2} ions producing conformational changes in which the molecule becomes more compact. This could result from the formation of intramolecular Ca^{+2} bridges. However, in the presence of the strongly binding groups of phospholipids, such bridges may become intermolecular linking the lipid and protein components. The subsequent development of additional lipid-protein interactions of a different nature are, of course, not excluded by these considerations.

Suggestions for Future Investigation

The effects of Ca^{+2} on the conformation of prothrombin as indicated by positive difference spectra and the alteration of the sedimentation coefficient concentration dependence suggest that further investigation of the effects of Ca^{+2} on prothrombin would be an important avenue of future research. The approaches possible include quantitation of Ca^{+2} -prothrombin binding by equilibrium dialysis and elucidation of the extent of the Ca^{+2} -induced conformational changes in prothrombin by spectrophotometric methods such as optical rotatory dispersion and circular dichroism. Physical methods such as viscosity measurements of prothrombin solutions coupled with further analysis of ultracentrifugation parameters such as \bar{M}_w , S, and D are advocated.

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APPENDIX

A simple program, SEDVEL, was developed for routine calculation of sedimentation coefficients using an APL/360 computer terminal.

The sedimentation coefficient is determined from the equation

$$S = \frac{1}{\omega^2 x} \cdot \frac{dx}{dt}$$

where x is the distance of the boundary in cm from the axis of rotation, t is the time in seconds, and ω is the angular velocity in radians per second.

Input consists of two vectors; a time vector A, consisting of the time (in order of increasing magnitude) at which each x was measured and a vector B, consisting of the corresponding x values measured from either the inner or outer reference hole on the rotor. If the measurements of x are taken at a constant time interval, A may be entered as this interval. Rotor speed must be specified in rpm as the constant RPM.

Output consists of a table organized under the headings of TIME, X (CM), LOG x and DEV, and the determined S observed value. The DEV column indicates the deviation of the entered values of $\log x$ from the values obtained by a least-mean-square fit of the data.

The program is shown below.

```

      ▽ SEDVEL [0]▽
    ▽ A SEDVEL B;M;SL
[1]  M←((ρB),4)ρ0
[2]  M[;2]←B
[3]  →6×1(B[ρB]>B[1])
[4]  M[;3]←10⊗7.33-B÷2.211
[5]  →7
[6]  M[;3]←10⊗5.72+B÷2.211
[7]  →10×1(ρ,A=1)
[8]  M[;1]←A
[9]  →11
[10] M[;1]←A+A×(1ρB)
[11] M[;4]←M[;3]-(A INCEPT M[;3])+A×SL←A SLOPE M[;3]
[12] '
      TIME (MIN)           X (CM)           LOG X           DEV
      '
[13] '
      S OBSERVED = ';2 RND SL×35001000000000÷RPM*2;' S'
    ▽

```

Three subprograms were used. The programs A INCEPT B and A SLOPE B determine, by the method of least mean squares, the intercept and slope of the log x versus t data. The subprogram N RND M is used to round off the S_{obs} value to 2 decimal places.

The constant 2.211 appearing in lines 4 and 6 of the program is the magnification factor of the measured x values and is due to the optical system of the ultracentrifuge. This value remained constant and when necessary, was changed by simple line-editing. Since rotor speeds were always in the vicinity of 60,000 rpm, the inner and outer reference hole distances of 5.72 and 7.33 cm were used.

B30045